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Experience-dependent Neuroplasticity in the Perilesion Cortex after Focal Cortical Infarcts in Rats

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Dedication

To my parents and lovely wife

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Experience-dependent Neuroplasticity in the Perilesion Cortex after Focal Cortical Infarcts in Rats

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The leading cause of long-term disability among adults in industrialized countries is stroke. Exploration of the brain mechanisms involved during recovery from stroke is likely to yield information that can be used to promote better functional outcome. After focal motor cortical infarcts, reorganization of movement representations in the remaining motor cortex has been linked to both spontaneous recovery and recovery induced by rehabilitative training. However, the mechanisms and nature of cortical reorganization remain poorly understood.

The central hypothesis of these dissertation studies is that synaptogenesis and structural reorganization in the cortex near the lesion are linked to spontaneous partial recovery and the beneficial effects of motor rehabilitative training after stroke-like injury. This was tested in a rat model of focal cortical ischemia by both behavioral and neuro-anatomical measures in perilesion cortex. In separate studies, it was found that motor

rehabilitative training on a skilled reaching task using the impaired forelimb after a unilateral ischemic lesion improved forelimb functional outcome and facilitated synaptogenesis in perilesion cortex. In addition, this improved functional recovery was disrupted by focal protein synthesis inhibition in perilesion cortex, suggesting the structural plasticity in this area plays an important role in regained function. Finally, it was also hypothesized that a therapy that enhances the efficacy of motor rehabilitation also enhances synaptic structural plasticity in perilesion cortex. Cortical electrical stimulation (CS) during motor rehabilitation has previously been shown to improve the efficacy of rehabilitation. Increased density of axodendritic synapses in perilesion cortex was found in rats that received cortical electrical stimulation of perilesion cortex during rehabilitation compared to rehabilitation alone, and the synaptic density was positively correlated with post-rehabilitation reaching performance. These findings suggest that CS-induced functional improvements may be mediated by synaptic structural plasticity in stimulated cortex.

Together these studies indicate that, after a cortical lesion in rats, motor rehabilitation alone or in conjunction with other efficacious therapies can greatly enhance synaptic structural plasticity in perilesion cortex. Furthermore, these studies suggest that rehabilitation induced improvements in functional outcome are dependent upon the structural and functional integrity of the reorganized perilesion cortex.

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Chapter 1: Introduction

1.1 Overview

Stroke is now the number one cause of long term disability in industrialized countries, and the population of stroke survivors continues to grow as a result of an increasing average life span and improved medical environments (American Heart Association, 2007). The loss of manpower due to disabilities caused by stroke and the heavy expenses of treatment and rehabilitation will continue to be a serious economic burden to modern society.

The major sequelae of strokes are contralateral impairments below the medullary decussation, which are followed by disuse of the impaired body side and compensatory overuse of the good side (*e.g.*, Schallert *et al.*, 1997; Hsu and Jones, 2005). The current standard treatment for functional recovery in a stabilized stroke patient is physical rehabilitation, an approach that has been based on empirical clinical evidence rather than scientifically modeled therapies (Wolf *et al.*, 2006; Luft and Hanley, 2006). Most patients want to regain the lost function of their impaired body side instead of having a hyperfunctioning ipsilateral side, but it is difficult to fully recover function in the impaired side even after physical rehabilitation. Knowing more about the underlying mechanisms by which brain structures respond to stroke and to subsequent rehabilitation would provide valuable knowledge which could then be applied during rehabilitation to maximize functional recovery in a stroke patient.

Reorganization of the remaining perilesion cortex following a cortical stroke is a possible mechanism of functional recovery that is supported by several research findings in rats (Castro-Alamancos and Borrell, 1995), monkeys (Friel *et al.*, 2000; Nudo *et al.*, 1996b), and humans (Leipert *et al.*, 2000; Green 2003). For example, after focal motor cortical infarcts, reorganization of movement representations in the remaining motor cortex has been linked to both spontaneous recovery (Conner *et al.*, 2005) and recovery induced by rehabilitative training (Castro-Alamancos and Borrell, 1995). However, the mechanisms and nature of cortical reorganization remain poorly understood. The purpose of my dissertation research is to explore both the neuroplastic effects of focal sensorimotor cortical (SMC) lesions on remaining cortex and how those effects relate to post-injury behavioral changes and functional outcome.

The central hypothesis of the dissertation is that the synaptogenesis and structural reorganization that occur in the cortex near the lesion are linked to spontaneous partial recovery and the beneficial effects of motor rehabilitative training after a stroke-like injury. This was tested by both behavioral measures and the quantification of neuro-anatomical changes in the perilesion cortex. The first study (Chapter 2) assessed the effects of motor-rehabilitation on both behavioral outcome and the structural reorganization of the perilesion cortex. The second study (Chapter 3) determined whether changes in the perilesion cortex are necessary contributors to the behavioral improvements driven by motor rehabilitation after cortical infarcts. The last study tested whether a therapy that enhances the efficacy of motor rehabilitation also enhances synaptic structural plasticity in the perilesion cortex.

The remainder of this chapter provides background information for the present studies, including lesion-induced changes in behaviors and neural plasticity, experience-induced neural plasticity in intact animals (*i.e.* the effect of motor learning) and in SMC lesion animals (*i.e.* the effect of motor rehabilitation), an overview of the anatomy of the sensorimotor cortex (SMC) in rats, and the use of anisomycin to determine the degree to which protein synthesis in particular brain subregions affects behavioral changes.

1.2 FOCAL UNILATERAL SMC LESIONS INDUCE CHANGES IN BEHAVIORS AND CORTICAL STRUCTURES.

1.2.1 Behavioral changes after unilateral SMC damage

Unilateral SMC lesions have been shown to result in a wide range of contralesional forelimb impairments that depend on the size, location, subcortical extent and temporal factors of the cerebral lesion. The most common results are paresis, sensory loss and learned non-use of the contralateral limb. Some examples include an increased reliance on the ipsilesional forelimb during upright exploratory movement in rats (Schallert *et al.*, 1997; Hsu and Jones, 2005) and functional deficits in skilled reaching performance in rats (Whishaw, 2000), monkeys (Nudo and Milliken, 1996), and humans (Green, 2003). Subtle ipsilesional impairments such as impaired dexterity of the hand (Sunderland *et al.*, 1999), deficits in wrist movement (Yarosh *et al.*, 2004), and reduced grip strength (Robinson *et al.*, 1990) in humans, as well as abnormal reaching movements in rats (Hsu and Jones, 2006), have also been found after unilateral SMC lesions.

Several studies have found that small lesions of the SMC enhance subsequent skilled learning in the ipsilesional forelimb in rats (Bury and Jones, 2002; Allred and Jones, 2004; Hsu and Jones, 2005), even in the presence of ipsilesional deficits (Hsu and Jones, 2006). Though reliance on the ipsilesional body side may facilitate behavioral compensation following strokes, it might also make animals more prone to neglect the impaired forelimb, contributing to the phenomenon of learned non-use (Taub *et al.*, 2002).

1.2.2 Bilateral cortical plasticity after unilateral SMC damage

Focal ischemic damage to the cortex has been shown to initiate several cellular and structural events that occur in both the lesioned and intact hemispheres (Keyvani and Schallert, 2002). Dendritic growth (Li *et al.*, 1998), increases in excitability (Que *et al.*, 1999) and increases in growth factors (*e.g.*, Hsu *et al.*, 1993; Kokaia *et al.*, 1998; and Lin *et al.*, 1997) are found in the lesioned hemisphere. Several studies have also investigated the neuroplastic events that occur in the contra-to-lesion cortex, such as dendrite addition (Adkins *et al.*, 2004; Jones and Schallert, 1992; Jones *et al.*, 1999; Biernaskie and Corbett, 2001; Stroemer *et al.*, 1995), synaptogenesis (Luke *et al.*, 2004; Hsu and Jones, 2005; Stroemer *et al.*, 1995), increased excitability (Que *et al.*, 1999; Witte *et al.*, 2000), and trophic factor expression (McNeill *et al.*, 1999).

Neuronal reorganization in the cortex has been shown to occur after strokes, such as the enlargement or shift of motor cortical representations in the affected hemisphere (Chollet *et al.*, 1991; Weiller *et al.*, 1993; Cramer *et al.*, 2000). Dijkhuizen *et al.* (2001) found that, at 14 days after stroke, electrical stimulation of the impaired forelimb in rats

leads to significant activation responses in the peri-infarction area as revealed by fMRI, and some studies (Liepert *et al.*, 2000; Green, 2003) suggest that the reorganization of motor cortical representations plays an important role in functional recovery following a stroke-like lesion. Conner *et al.* (2005) also showed that motor representations were reorganized and enlarged in the perilesion cortex following a focal SMC lesion. Though the neuronal basis of plasticity mediating cortical map reorganization is still poorly understood, Kleim *et al.* (2003a) found that the circuitry of the motor cortex in adult rats is labile and requires continuous protein synthesis to maintain its functional organization. It is unknown to what extent the plasticity of synaptic connectivity contributes to these examples of cortical map plasticity following cortical infarcts.

1.3 EXPERIENCE-INDUCED CHANGES IN THE CEREBRAL CORTEX

1.3.1 Experience-induced changes in intact animals

Many studies have demonstrated that behavioral experiences, such as increased environmental interaction or motor learning, are associated with major structural and functional changes in the cortex of intact animals (*e.g.*, Nudo, 2003; Rioult-Pedotti *et al.*, 2000; Butefisch, 2004). Some environmental experiences, such as increasing the complexity of the environment during the developmental period or adulthood in rats, have been shown to result in structural changes in the visual cortex, including increases in cortical volume (Diamond *et al.*, 1964), dendritic arborization (Greenough and Volkmar, 1973), dendritic spine density (Globus *et al.*, 1973), synapses per neuron

(Turner and Greenough, 1985), and multisynaptic boutons (Jones *et al.*, 1997) compared to animals that received standard laboratory housing.

1.3.2 Motor learning induces structural and functional plasticity in the motor cortex in intact animals.

Both acrobatic training and skilled reach training are examples of motor learning that alter the structural organization of the motor cortex. Acrobatic training, which requires a rat to traverse a series of challenging obstacles (*i.e.*, climbing a chain rope and climbing a ladder), increases synapse number per neuron in layer II/III (Kleim *et al.*, 1996) and V (Jones *et al.*, 1999) of the motor cortex and synapse number per Purkinje cell in the paramedian lobule of the cerebellum (Black *et al.*, 1990) compared to animals receiving simple repetitive exercise.

Skilled reach training is the task used in my dissertation for motor rehabilitation and assessment of motor function. In skilled reach training, rats are required to learn to extend a forelimb through an opening in order to grasp and retrieve a food pellet for reward. Following training on this task in intact rats, increases have been found in pyramidal neuron dendritic arborization in layers II/III and V (Greenough *et al.*, 1985; Withers and Greenough, 1989) and synapse number per neuron in layer V (Kleim *et al.*, 2004) of the motor cortex contralateral to the trained forelimb compared to the motor cortices of unskilled training controls.

Skilled training also induces the reorganization and expansion of movement representation maps in the motor cortex contralateral to the trained wrist and digits in rats (Kleim *et al.*, 1998) and monkeys (Nudo *et al.*, 1996a), as revealed by intracortical

microstimulation mapping. In contrast, no expansion of motor maps is found in unskilled training controls (Kleim *et al.*, 1998) or in animals that receive exercise training (Kleim *et al.*, 2002). Through the use of functional magnetic resonance imaging (fMRI), changes in activation patterns in the motor cortex have been linked to skilled acquisition in humans (*e.g.*, Karni *et al.*, 1998; Ungerleider *et al.*, 2002). Transcranial magnetic stimulation (TMS) has revealed that changes in movement representations are associated with skilled reaching in humans as well (*e.g.*, Muellbacher *et al.*, 2001; Pascual-Leone, 1995; Perez *et al.*, 2004). In rats, the reorganization of motor representation maps coincides with increased synapse numbers per neuron in the motor cortices of the same animals (Kleim *et al.*, 2002; 2004) after sufficient time and practice on the task (*e.g.*, 10 days in Kleim *et al.* 2004), though maps have the capacity to change rapidly (Jacob and Donoghue, 1991; Nudo *et al.*, 1990).

Most of the effects of reach training on cortical plasticity have been found in the cortex contralateral to the trained limb, whereas subtle changes in dendritic morphology have also been found in the cortex ipsilateral to the trained forelimb (Greenough *et al.*, 1985). In humans, Cramer *et al.* (1999) showed ipsilateral cortical activation (as revealed by fMRI) during unilateral distal forelimb movements, and also that the area of activation was spatially distinct from that activated during contralateral distal forelimb movements.

In addition to the findings above which suggest that motor learning induces major structural and functional neuroplastic responses in the motor cortex, lesions or reversible inactivation of the SMC in rats (*e.g.*, Whishaw *et al.*, 1986), cats (Martin and Ghez, 1991), and monkeys (Pavlides *et al.*, 1993) have been shown to impair skilled reaching

in the contralateral forelimb. These findings strongly support the idea that neural plasticity in the motor cortex plays an important role in motor skills learning.

1.3.3 Experience-induced changes following SMC lesions

Many of the findings mentioned in the previous section support the idea that behavioral experiences can alter structural and functional plasticity in the cortex in intact animals. It is reasonable to hypothesize that behavioral experiences or manipulations following a SMC lesion will use many of the same mechanisms to drive structural and functional changes in the remaining cortex and will also affect behavioral recovery. The behavioral recovery of experimental animals with brain injury is affected by experiences such as exposure to complex environment housing (Rose *et al.*, 1993; Galani *et al.*, 1997) or acrobatic training (Jones *et al.*, 1999). In addition, motor rehabilitation can significantly enhance motor function depending on the severity of the impairments (Duncan *et al.*, 2000).

1.3.3.1 Effect of motor rehabilitative training following SMC lesions

It is generally believed that stroke patients who receive physical rehabilitation have better functional recovery. Focal cortical lesions in rats (Whishaw, 2000), monkeys (Nudo and Milliken, 1996), and humans (Green, 2003) result in impairments in skilled reaching performance. With subsequent rehabilitative training in skilled reaching, reorganization of motor representations in the perilesion cortex is induced and is thought to contribute to relevant functional recovery in rats (Castro-Alamancos and Borrell, 1995), monkeys (Friel *et al.*, 2000; Nudo *et al.*, 1996), and humans (Leipert *et al.*, 2000; Green 2003).

Ablation of the reorganized cortex in rats (Castro-Alamancos and Borrel, 1995; Conner *et al.*, 2005) or inactivation of the perilesion cortex by disruptive transcranial magnetic stimulation (TMS) in humans (Fridman *et al.*, 2004) reinstates the functional deficit. These studies suggest that functional reorganization in the perilesion cortex after rehabilitative training plays an important role in the functional recovery of the impaired limb. However, these previous studies focused on the electrophysiological mapping of motor cortical representations. It has been found in non-human primates that a focal ischemic lesion in the primary motor cortex hand area results in extensive ipsilateral axonal sprouting from the ventral premotor cortex to the somatosensory hand area (Dancause *et al.*, 2005). It has also been reported that acrobatic training after a focal SMC lesion prevents a loss of cortical volume in the hindlimb SMC adjacent to the lesion compared to lesioned rats who receive only simple exercise (Chu and Jones, 2000). The findings mentioned above all suggest that the functional recovery induced by motor rehabilitative training might be related to structural plasticity in the perilesion cortex. The possibility that motor rehabilitative training contributes to synaptogenesis in the perilesion cortex needs further investigation. Perforated (Perf) synapses and synapses formed by multisynaptic boutons (MSBs) are two synapse subtypes have been linked with increases in synaptic efficacy (*e.g.*, Geinisman *et al.*, 1991; Geinisman *et al.*, 2001) and have previously been found to be increased in the motor cortex in association with the acquisition of motor skills (Kleim *et al.*, 1998; Jones *et al.*, 1999). Increases in perforated synapses and multisynaptic boutons have been found in the striatum after cortical lesions in relation to reactive synaptic sprouting (McNeill *et al.*, 2003). Toni *et al.* (1999) also showed that the induction of long-term potentiation resulted in an

increased proportion of MSBs in hippocampus. It has been found that the number of AMPA receptors, the measurement of which plays an important role in determining synaptic efficacy (*e.g.* Renger *et al*., 2001), was significantly increased in perforated synapses compared to other axospinous synaptic junctions (Ganeshina *et al*., 2004); whereas a reduction in the size of perforated postsynaptic densities (PSD) in the hippocampus has been found in aged rats with spatial learning impairments (Nicholson *et al.*, 2004). The mechanisms that cause perforations are still unknown. Some studies suggest that synaptic perforations are not permanent features but occur transiently in response to enhanced synaptic activity (Sorra et al., 1998). The effect of motor rehabilitative training on those two efficacious synapses subtypes in the perilesion cortex also needs further investigation.

Rehabilitation has been shown to contribute to behavioral recovery and functional plasticity in the remaining motor cortex after damage to the SMC. Combining rehabilitation with other therapies may further enhance experience-induced behavioral recovery and neural plasticity. Biernaski and Corbett (2001) found that rats receiving daily reach training combined with enriched environment exposure following a SMC lesion showed greater improvement in reaching performance. Rehabilitation combined with electrical stimulation of the perilesion cortex has also been shown to enhance the effects of rehabilitation in rats (Adkins-Muir and Jones, 2003; Kleim *et al.*, 2003b; Teskey *et al.*, 2003), monkeys (Plautz *et al.*, 2003), and humans (Brown *et al.*, 2006). The possibility that cortical stimulation paired with motor rehabilitative training contributes to synaptogenesis in the perilesion cortex needs further investigation as well.

Current evidence suggests that both SMC lesions and experiences such as rehabilitation result in behavioral changes and neuroplastic responses. Because the present dissertation focuses on structural plasticity, a more detailed account of the anatomical organization of the sensorimotor cortex is relevant to these studies.

1.4 ANATOMY OF THE SENSORIMOTOR CORTEX IN RATS

1.4.1 Electrophysiological and cytoarchitectural characteristics of the sensorimotor cortex in rats

The sensorimotor cortex (SMC) in rats consists of the primary sensory cortex (SI) and motor cortex (MI). MI is anterior and medial to SI but, unlike humans, the forelimb representation areas of MI and SI are only partially separate, the hindlimb areas completely overlap, and the face areas are completely separate (Hall and Lindholm, 1974; Wise and Jones, 1977; Donoghue and Wise, 1982). Both SI and MI are distinctive in electrophysiological and cytoarchitectural characteristics. Electrophysiologically, MI is delineated by discrete body movements elicited by electrical stimulation of the cortex whereas SI is characterized by receptive fields that respond to light cutaneous stimulation (Hall and Lindholm, 1974; Donoghue and Wise, 1982). Cytoarchitecturally, MI is characterized by large pyramidal neurons in layer V and the lack of a clearly defined granular layer IV, whereas SI contains clusters of granular cells interspersed with dysgranular strips in layer IV and small to medium sized pyramidal neurons in layer V (Donoghue and Wise, 1982; Welker *et al.*, 1984; Sanderson *et al.*, 1984). The region of overlapping forelimb representation areas, which can be identified cytoarchitectonically

by the clusters of granular cells in layer IV and large pyramidal neurons in layer V (Donoghue and Wise, 1982), was targeted for the unilateral ischemic lesions that I used in my dissertation. These lesions have been shown to consistently cause impairments in the contralateral forelimb in rats (Luke *et al.*, 2004; Allred and Jones, 2004; Hsu and Jones, 2005).

1.4.2 Subcortical and intracortical connections of the SMC

1.4.2.1 Subcortical connections of the SMC

Most of the connections between the SMC and subcortical areas are reciprocal. Both MI and SI receive subcortical afferent connnections from the thalamus. In the SI, granular (layer IV and lower layer III) and dysgranular areas receive input from the thalamus whereas projections from the thalamus mainly terminate in layer III and I in MI (Killackey, 1973; Killackey and Sherman, 2003). In the overlapping area of MI and SI, the projections from the ventrolateral nucleus of the thalamus, which receives inputs from ascending spinal pathways, the deep cerebellar nuclei, and the basal ganglia, mainly terminate in layer II, III and V (Donoghue and Parham, 1983).

Both SI and MI send projections to many subcortical areas. SI sends projections to subcortical areas which are involved in the modulation of motor performance, including the striatum (McGeorge and Faull, 1989; Donoghue and Parham, 1983), superior colliculus (Wise and Jones, 1977), and pontine nuclei (Wiesendanger and Wiesendanger, 1982; Mihailoff *et al.*, 1985). The majority of the corticoefferent projections that originate in layer V pyramidal neurons in MI travel descendingly to the spinal cord (Leong, 1983; Bates and Killackey, 1984; Miller, 1987) and pons (Legg *et al.*,

1989), and also collaterally project to the striatum (Donoghue and Kitai, 1981), basilar pontine nucleus (Mihailoff *et al.*, 1985), and the reticular formation (Valverde, 1966). Layer V pyramidal neurons in the overlapping region have been shown to make direct monosynaptic connections with motor neurons (Valverde, 1966; Hicks and D'Amato, 1977).

1.4.2.2 Intracortical connections of the SMC

MI receives the majority of the ipsicortical projections from SI, SII and the medial agranular cortex (Donoghue and Parham, 1983). The projections from SI mainly originate from layers II, III, V, and VI in the dysgranular field, a cytoarchitecturally distinct region of SI, and also from layers V and VI of the densely granular field, which is the part of SI that is strongly activated by cutaneous inputs (Donoghue and Parham, 1983). Within MI in monkeys, there are many axonal projections from pyramidal cells in layers II and III to pyramidal cells in layer V (Asanuma and Rosen, 1972). MI also projects directly to SI, the efferents arising mostly from layer V and less so from layer III (White and DeAmicis, 1977) in the mouse. These efferents from MI mainly terminate in dysgranular strips in SI in rats (Chapin *et al.*, 1987). MI and SI were expected to have projections with each other within their overlapping regions while also having ipsilateral cortical connections in the non-overlapping regions (Fabri and Burton, 1991). In addition to ipsilateral connections, both SI and MI receive abundant transcallosal connections, primarily excitatory, that mainly terminate in layers II and III (Chapin *et al.*, 1987). By injections of biotinylated dextran amine (BDA), an anterograde tracer, in

overlapping regions of MI and SI, Bury *et al.* (2000) also showed transcallosal projections that terminate in the contralateral motor cortex.

1.5 EFFECT OF ANISOMYCIN ON MOTOR LEARNING

Many studies have shown that protein synthesis is required for learning and memory. Davis and Squire (1984) showed that protein synthesis inhibition (PSI), either during or shortly after training, inhibits the formation of long-term memory, suggesting that PSI affects both the acquisition and retention-consolidation phases of learning. Anisomycin is an antibiotic produced by *Streptomyces griseolus* which inhibits protein synthesis by interfering with peptidyl transferase activity in eukaryote ribosomes (Grollman, 1967). Focal anisomycin injections into the hippocampus have been shown to result in the inhibition of spatial learning and memory (*e.g.*, Morris *et al.*, 2006; Naghdi *et al.*, 2003), whereas injections into the nucleus accumbens block the early consolidation of instrumental learning (Hernandez *et al.*, 2002) in rats. Focal anisomycin injection has also been used for reversible inactivation of amygdala to impair fear conditioning (Sacchetti *et al.,* 2007).

It was recently shown that focal injections of anisomycin in the SMC in rats abolish motor skill learning (Kleim *et al.*, 2003a; Luft *et al.*, 2004). Li *et al.* (2001) also recently showed that neuroplasticity in the motor cortex is associated with motor learning in monkeys, and that such plasticity in the motor cortex may also enhance recovery after brain lesions (*e.g.*, Hallett, 2001). Since motor learning can be blocked by PSI in the motor cortex, it is reasonable to hypothesize that PSI can block neuronal plasticity after a

brain lesion as well. In addition, Kleim and colleagues (2003a) showed that PSI by anisomycin injection in the SMC not only causes skilled movement impairments and a long term loss of the motor representation map, but also decreases synapse number and size. The findings above suggest that anisomycin could be used to inhibit protein synthesis and thereby disrupt structural plasticity in the perilesion cortex in lesion animals.

Anisomycin injections have been used in animal experiments for decades. In some experiments, microinfusions of anisomycin into the targeted brain area have been used (Nader *et al.*, 2000; Kleim *et al.*, 2003a; Canal *et al.*, 2007) while peripheral injections have been used in others (*e.g.*, Lattal and Abel, 2004; Suzuki *et al.*, 2004). Peripheral administration might not be useful in my dissertation because we would have no way to determine whether the behavioral changes that occured after anisomycin injection were due solely to PSI in the desired target region. Furthermore, peripheral injections might cause unwanted behavioral effects such as transient sickness. The focal injection of anisomycin directly into targeted brain regions does have some limitations though. The injection itself causes mechanical tissue damage and the diffusion of drugs might be uneven throughout the targeted brain area. We can rule out behavioral or structural changes caused by mechanically injured tissue by injecting a vehicle solution of artificial cerebrospinal fluid (ACSF) into control animals, and we can decrease the uneven diffusion of anisomycin by employing a steady and slow injection pace. Even with these potential shortcomings, the focal injection of anisomycin into the targeted brain region is a more appropriate method of anisomycin administration than peripheral injections for the experimental questions of my dissertation.

Chapter 2: Motor-rehabilitation using the impaired forelimb after a unilateral ischemic lesion improves behavioral outcome and facilitates structural reorganization of the perilesion cortex in rats

2.1 ABSTRACT

After focal motor cortical lesions in rats and monkeys, reorganization of motor representations can be induced by motor rehabilitative training. The purpose of this study was to determine whether functional improvements resulting from motor rehabilitation after cortical infarcts are linked with synaptic structural reorganization of the perilesion motor cortex. After training to a plateau on a unilateral skilled reaching task with one limb, rats received either unilateral ischemic (endothelin-1 induced) lesions of the contralateral sensorimotor cortex or sham operations. Lesion and sham groups then received either training with the impaired forelimb on the skilled reaching task for 4 consecutive weeks or no-training control procedures. Lesion animals that received rehabilitative training had much greater functional recovery of skilled reaching performance than those with no training. Synaptic density in layer V of the perilesion motor cortex (remaining medial and lateral agranular regions) was estimated using the physical disector method for quantitative transmission electron microscopy. Rehabilitated rats with lesions, but not sham-operates, had a significantly greater axodendritic synaptic density compared with untrained controls. Rehabilitation increased synaptic density in lesion rats by 12% and 18% compared with lesion rats

receiving no rehabilitation and training-matched sham animals, respectively. Furthermore, after lesions, rehabilitation increased presumed efficacious synapse subtypes, perforated synapses and multisynaptic boutons, by 54% and 67%, respectively, compared to lesion no-rehabilitation controls. These results indicate that rehabilitative skilled reaching with the impaired forelimb enhances functional recovery and the structural reorganization of the perilesion cortex.

2.2 INTRODUCTION

About 700,000 Americans experience a new or recurrent stroke each year, which approximates to someone in the United States having a stroke every 45 seconds. Mortality from stroke in 2002 was roughly 273,000, indicating that approximately 400,000 people survived their strokes with an accompanying long-term disability. In addition, the estimated costs of stroke for 2007 will be $62.7 billion and the mean lifetime cost for a person in the USA who suffers from a single ischemic stroke is about $140,000 (American Heart Association, 2007). The loss of manpower due to disabilities caused by stroke and the heavy expenses of treatment and rehabilitation will continue to be a serious economic burden to modern society.

The major disabilities caused by strokes are contralateral impairments followed by disuse of the impaired body side and compensatory overuse of the good side (*e.g.*, Schallert *et al.*, 1997; Hsu and Jones, 2005), and physical rehabilitation is the current standard treatment for functional recovery in stroke patients. Though most patients want to regain the lost function of the impaired body side instead of having a hyperfunctioning ipsilateral side, it is difficult to fully recover function in the impaired

side even after physical rehabilitation. Thus, there is a major need to understand more about the underlying mechanisms by which brain structures respond to stroke and to rehabilitation.

Many studies indicate that reorganization of the remaining perilesion cortex following a cortical stroke is a possible mechanism of functional recovery. For example, after focal motor cortical infarcts, impairments in skilled reaching performance have been found in rats (Whishaw, 2000), monkeys (Nudo and Milliken, 1996), and humans (Green, 2003). Following rehabilitative training in skilled reaching, reorganization of motor representations in the remaining motor cortex is induced and this is thought to contribute to relevant functional recovery in rats (Castro-Alamancos and Borrell, 1995), monkeys (Friel *et al.*, 2000; Nudo *et al.*, 1996b), and humans (Leipert *et al.*, 2000; Green 2003). However, these previous studies focused on the electrophysiological mapping of motor cortical representations and, thus, the mechanisms and nature of cortical reorganization remain poorly understood. It has been found that a focal ischemic cortical lesion results in extensive axonal sprouting (Dancause *et al.*, 2005) and increases in plasticity related proteins (*e.g.* GAP-43, review in Carmichael, 2003) in the perilesion cortex. It has also been reported that acrobatic training after a focal SMC lesion prevents a loss of cortical volume in the hindlimb sensorimotor cortex adjacent to the lesion compared to lesioned rats who received only simple exercise (Chu and Jones, 2000). The possibility that motor rehabilitative training facilitates synaptogenesis in the perilesion cortex needs further investigation.

The purpose of the present study was to determine whether motor-rehabilitation using the single pellet skilled reaching task can enhance behavioral recovery and can

contribute to synaptic structural reorganization of the perilesion cortex. All rats, 3-4 months old, were trained to a plateau on a skilled reaching task with their preferred forelimb. Sham surgeries were performed or unilateral focal ischemic lesions were made in the forelimb representation region of the SMC contralateral to the preferred forelimb of each animal. After surgeries, both lesion and sham animals were subdivided and placed into either a rehabilitation group or a control group that did not receive rehabilitation. Skilled reach training was used as a rehabilitative training task and probe trial tests were held once a week over a 4-week span. Performance with the impaired forelimb was evaluated by success rates and movement patterns. Perilesion cortical tissue was processed for electromicroscopic measurement of synaptic structural plasticity using stereological methods.

2.3 MATERIALS AND METHODS

2.3.1 Subjects and experimental designs

Thirty-four adult 3 to 4 month old male Long-Evans hooded rats were gently handled beginning after weaning. The rats were housed in pairs in transparent cages on a 12:12 hour light:dark cycle and received water *ad libitum*. Rats were placed on scheduled feeding (15g, once per day) to ensure rats were not sated at the time of testing. All animal use was in accordance with a protocol approved by the Animal Care and Use Committee of the University of Texas at Austin. Following pre-operative single pellet skilled reach training with the preferred forelimb, animals were randomly divided into Lesion and Sham groups with the exception that they were matched as closely as possible for pre-operative skilled reaching performance. Animals then were further subdivided based upon their initial post-operative reaching performance so that there were four groups: (1) lesion + rehabilitation (Les Rehab), n = 9, (2) lesion + no rehabilitation control (Les CTL), n = 8, (3) sham + rehabilitation (Sham Rehab), n = 9, (4) sham + no rehabilitation control (Sham CTL), n = 8. Animals in both no rehabilitation control groups were placed into skilled reaching boxes for the same period of time as the animals in the rehabilitation groups but with food pellets provided on the floor.

2.3.2 Surgical procedure

Animals underwent sham operations or unilateral ischemic sensorimotor cortical lesions by topical administration of endothelin-1 (ET-1; Adkins *et al.*, 2004; Fuxe *et al.*, 1997). This lesion method has previously been found to produce focal damage to cortical layers

I to VI underlying the craniectomy, depending on the dosage of ET-1 (Hsu and Jones, 2005; 2006), and this infarct method also enables precise control of cortical lesion boundaries (*e.g.* relative to middle cerebral artery occlusion methods). Rats were anesthetized with a cocktail of ketamine (90-100mg/kg) and xylazine (8-9 mg/kg). The lesions were aimed at the overlapping primary somatosensory and primary motor cortical representation regions of the forelimb (Donoghue and Wise, 1982) in the cortex opposite to the preferred-for-reaching forelimb. After removing the skull and dura between 1.5 mm posterior and 2.5mm anterior to bregma, and between 3.0 and 4.5 mm lateral to midline, lesions were produced by placing 2.5 µl of endothelin-1 (80µM, 0.2µg/µl in sterile saline) directly onto the pial surface. Endothelin-1 was applied in 2 drops (1.5 and 1.0 µl each) that were 2 min apart and the surgical site was left undisturbed for 10 min after the last drop. Gelfoam was then placed on the cortex to fill the craniectomy. Sham-operated rats received all procedures up to, but not including, skull removal to avoid the production of behavioral and neurochemical asymmetries associated with craniectomies (Adams *et al.*, 1994).

2.3.3 Reach training method

The single pellet-retrieval task was performed using an apparatus adapted from those used by McKenna and Whishaw (1999), Miklyaeva and Whishaw (1996), Peterson and Devine (1963) and Withers and Greenough (1989). The clear Plexiglas apparatus was 26 cm long by 35 cm high by 16cm wide with a 1 cm wide and 23 cm high window in the center of one wall (Figure 2.1). A 3 cm tall exterior shelf was adhered outside the window. Animals were trained to reach for pellets (45 mg banana-flavored food pellets;

Bioserve, Frenchtown, NJ) from a well in the shelf at a distance of 1 cm from the window. As shown in Figure 2.1, the reaching apparatus effectively forced rats to use the trained forelimb for pellet retrieval. A 2 mm diameter metal rod was attached to the shelf in front of the reaching window to prevent animals from scraping pellets into the chamber or using their tongue to retrieve them.

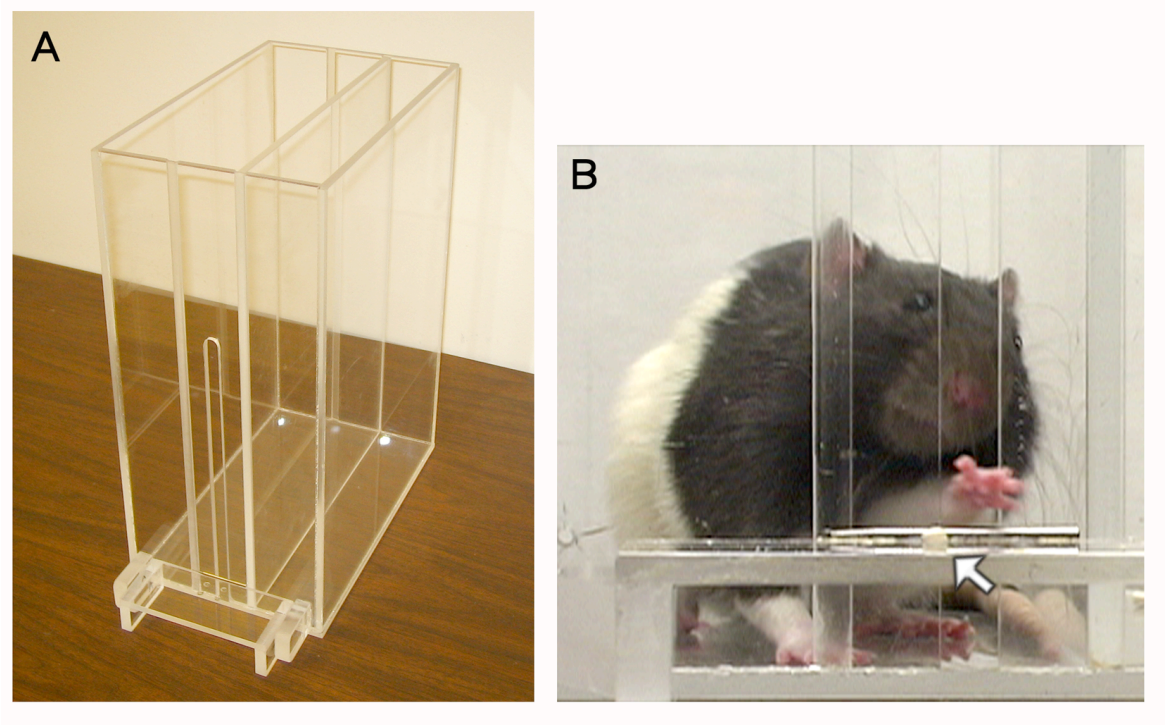

Figure 2.1 Reach training apparatus.

(A) Rats were trained to reach for food pellets through the 1 cm wide window. A Plexiglas wall was placed into the apparatus ipsilateral to the animal's trained limb and pellets were placed in the well cater-corner to this limb. This configuration effectively forced rats to use the trained forelimb for pellet retrieval (B). The white arrow indicates a food pellet.

Animals were given food pellets in their home cages prior to the experiment to reduce neophobic responses. Body weights changed from week to week, though this was kept between 90 and 115% of initial body weight during the experiment. All rats were initially given a shaping period (3 to 4 days) which consisted of being placed for 20 min in the chamber, with their cagemate on day 1 and alone beginning on day 2, without the interior wall and with food pellets placed on the floor. On day 3, the food pellets were put on the shelf in a central position and rats were allowed to reach with either forelimb. Nearly all rats develop a preference for using one forelimb in unilateral reaching tasks (Whishaw, 1992). Once the rats demonstrated a preference for a limb (using this limb in >70% of reaches) and made >20 reaches in 20 min, then pre-operative shaping ceased and the rats received 7-10 days of pre-operative reach training (30 reaching trials per day) with their preferred limb and reached the criterion of making at least 40% successful reach attempts with this limb by the last day of training. Pre-operative training was performed to ensure equivalency of reaching proficiency and rate of improvement between groups (which was used to match groups). Two rats were omitted from the study because they failed to meet the pre-operative criterion of 40% successful reach attempts using their preferred forelimb.

Rats received lesions in the hemisphere opposite to the preferred forelimb or sham operations 1-2 days after the end of pre-operative training. Post-operative rehabilitation training and/or probe trial testing was with the pre-operatively preferred limb and, in lesion groups, the impaired forelimb. All animals received one probe test (probe 1), which consisted of 10 reaching trials, 4 days after surgeries and once a week for 4 weeks

(probe 2-5). Both lesion and sham animals were then further grouped into either rehabilitation (Les Rehab and Sham Rehab) or no rehabilitation control groups (Les CTL and Sham CTL). The two rehabilitation groups then received rehabilitative training beginning one day after each probe trial for 4 weeks (*i.e.* each week consisting of 5 consecutive training days, a probe trial on the following day, and then a day off). Daily rehabilitative training sessions consisted of 60 reaching trials or 20 minutes, whichever came first. For each trial, a single pellet was placed in a well and rats were allowed up to 5 reach attempts until the pellet was either grasped or knocked from its well. Successful reaches were defined as the animal grasping the food pellet with its forepaw and bringing it directly to its mouth and eating it. Unsuccessful reach attempts included "misses", in which the pellet was missed or knocked from the well, and "drops", in which the pellet was successfully grasped, but dropped before eating. The percentage of successful retrievals, drops, and misses per reach attempt were measured each day.

2.3.4 Analysis of reaching movements

Reaching movements were quantified using an adaptation of a rating scale developed by Whishaw and others (*e.g.*, Whishaw *et al.*, 1993; Metz and Whishaw, 2000). This was used to analyze reaching movements one day prior to surgeries (Pre-OP), one day prior to rehabilitation (Post-OP), and one day following rehabilitation (Post-Rehab). The Whishaw rating scale is based on Eshkol-Wachmann movement notation and has been found to be sensitive to forelimb impairments after motor or lateral-frontal lesions in rats (Gonzalez *et al.*, 2004). The use of an inner chamber wall in the present study invalidates a subset of the movements analyzed with this scale (elbow to midline, digits

to midline, and arpeggio: pronation of the wrist) and these movements were omitted from the analysis. Seven components were analyzed: (1) Aim: the elbow is adducted and the forelimb is aligned with the slit of the chamber. (2) Advance: the head is lifted and the limb is advanced directly toward the pellet. (3) Digits open: the digits are extended and opened at the end of the advance. (4) Grasp: closure of the digits to secure the pellet. (5) Supination 1- the paw is dorsiflexed and supinated 90° as the limb is withdrawn. (6) Supination 2- the paw is supinated again by approximately 45° to bring the pellet to the mouth. (7) Release: the digits are opened and the pellet is released into the mouth. For each animal, five successful reaches were recorded using a digital video camera and then each reach was analyzed frame by frame. Observations of abnormal movements were recorded, including movments that were either absent (compensated for entirely by other body movements), unrecognizable, or recognizable but slightly abnormal. The rater was blind to the experimental group condition. Representative examples of abnormal movements are shown in Figure 2.2.

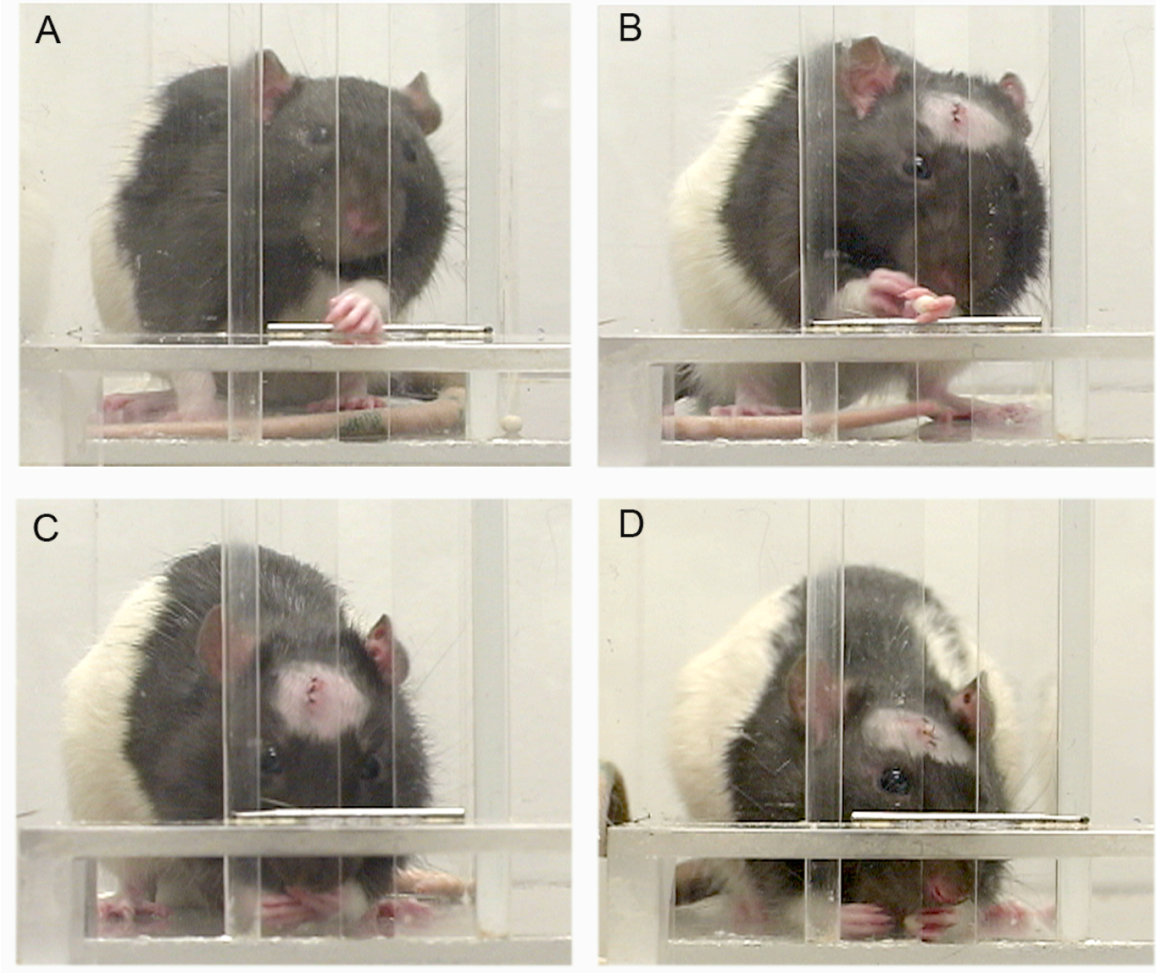

Figure 2.2 Representative examples of reaching movements.

Rats normally grasp the pellet securely in the paw (A), supinate the wrist during pellet withdrawal and then release the pellet into the mouth using paw and digits (C). The examples of abnormalities in these movements include abnormal grasping (B), failure to supinate and trying to use the mouth to release the pellet from the paw (D).

2.3.5 Assessment of contralesional impairments by forelimb placement during locomotion

The Footfault test was used to assess coordinated forelimb placement during locomotor movements (Barth *et al.*, 1990). Animals were placed on an elevated grid platform with 10.89 and 4.84 cm^2 grid openings and videotaped for 2 min. Animals were recorded once before surgery, one day before rehabilitation (3 days after surgery), and one day after rehabilitation. Errors were measured as slips with either forelimb through the grid openings. The number of forelimb steps and the number of errors were assessed using slow-motion video playback. Data shown are % contralesional forelimb errors per step.

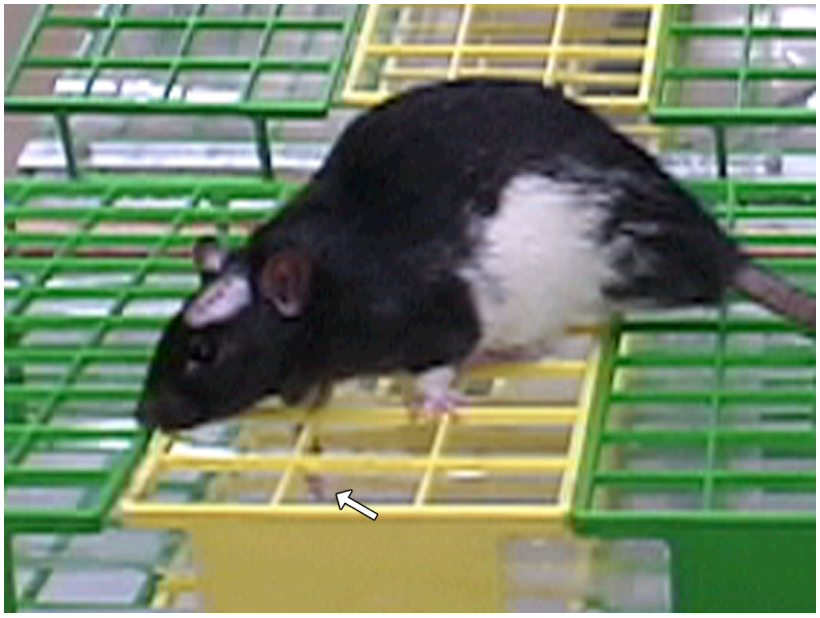

Figure 2.3 Footfault test.

Animals were placed on the elevated grid platform and errors were measured as slips with either forelimb through the grid openings. The arrow indicates a slip with the impaired limb.

2.3.6 Histological methods

One day after the last probe trial session, animals were anesthetized with a lethal dose of sodium pentobarbital and perfused intracardially with 0.1M phosphate buffer followed by fixative solution (2% paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M phosphate buffer). Alternating sets of 200, 100 and two 50 µm-thick sections of the cerebrum were cut using a Leica VT1000S vibratome within 24 hours after perfusion. The 50 µm sections were collected and then stained with Toluidine blue for lesion reconstruction and volume measurement. For electron microscopic analysis of synaptic density and light microscopic measures of neuronal density, non-necrotic/non-gliotic tissue in the peri-lesion motor cortical region, inclusive of the medial and lateral agranular region between 1.2 and 1.6 mm anterior to bregma, was sampled. Using a stereomicroscope, this region was identified, using macrostructural landmarks and unique cytoarchitectural characteristics which are evident in unstained tissue (Jones *et al.*, 1999), and was removed in the 200 µm sections (Figure 2.4). All samples were then placed in cacodylate-buffered osmium tetroxide, and *en bloc* stained with 2% uranyl acetate for 45 min. Samples were then dehydrated and sandwich-embedded in Eponate-12 resin. Semithin sections (0.8 µm thickness) were then extracted, stained with Toluidine Blue and used to estimate neuronal density and to more precisely localize the region for electron microscopic sampling. Serial silver gray ultrathin (70 nm) sections were obtained from the osmicated samples using a Leica Ultracut R microtome, mounted onto slotted copper grids coated with formvar film, and stained with lead citrate to be used for electron microscopic measures of layer V synaptic density. All histological data were collected with the experimenter blind to the experimental condition.

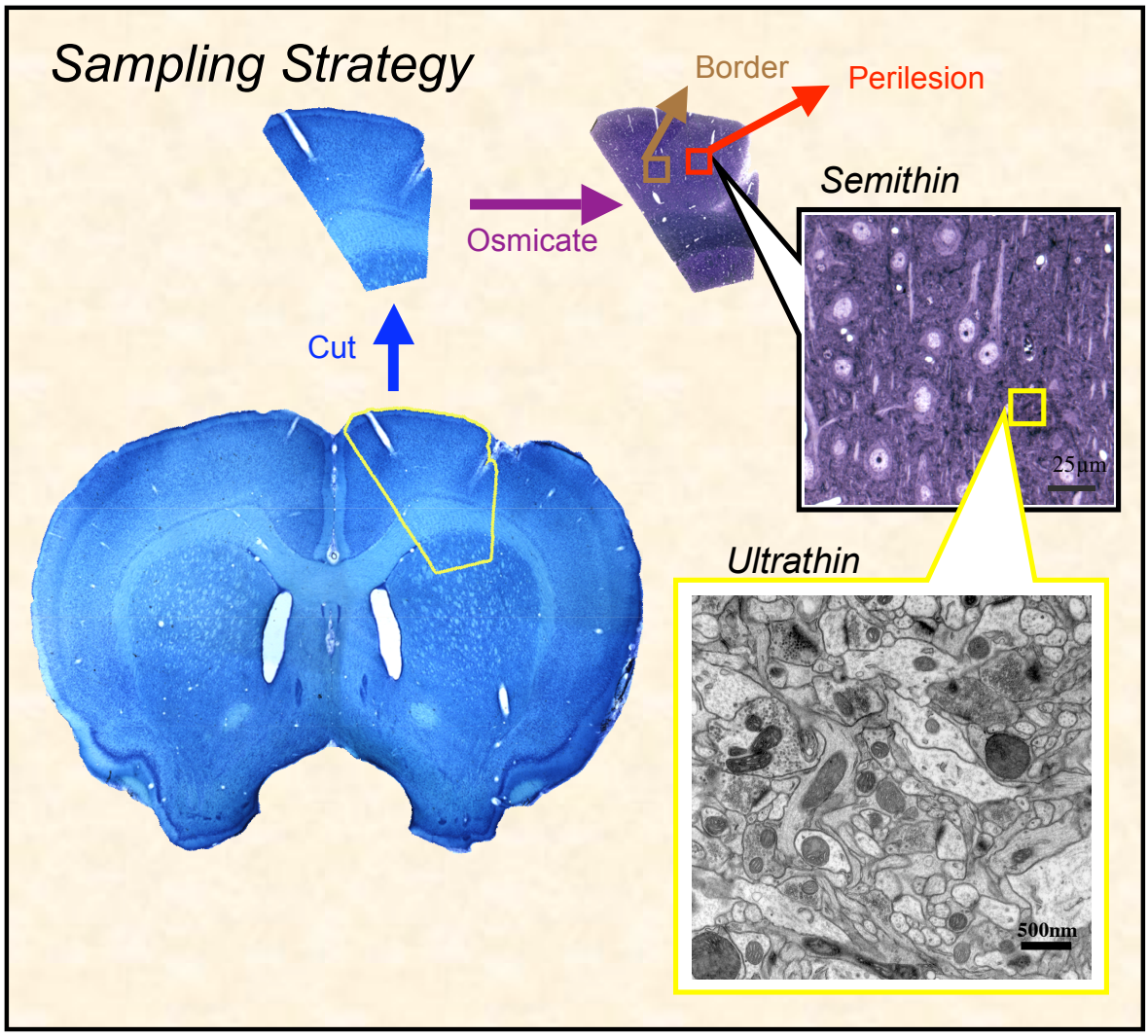

Figure 2.4 Sampling strategy.

Tissue in the perilesion motor cortical region was sampled inclusive of the medial and lateral agranular region between 1.2 and 1.6 mm anterior to bregma. All samples were placed in cacodylate-buffered osmium tetroxide, and *en bloc* stained with 2% uranyl acetate. Semithin sections (0.8 µm thickness) were then extracted, stained with Toluidine Blue and used to estimate neuronal density (in both "Perilesion" and "Border" subregions) and to more precisely localize the region for electron microscopic sampling. Serial silver gray ultrathin (70 nm) sections were obtained from the osmicated samples and stained with lead citrate to be used for electron microscopic measures of layer V synaptic density.

2.3.7 Lesion reconstruction and volume estimates

The extent of each lesion was reconstructed onto the left hemisphere of schematic coronal sections adapted from Paxinos and Watson (1986). The volumes of remaining cortex within the SMC region of the lesioned hemisphere and of the contralateral cortex were estimated to determine whether the tissue loss was similar in the two lesion groups. The sampling scheme focused on the region targeted by the lesion, starting with the appearance of the head of the caudate nucleus as the first of six sections spaced 800 μm apart. For infarcted cortex, the measurements included all remaining non-necrotic/non-gliotic cortical tissue. The volume within the SMC region was estimated using the Cavalieri method (Gundersen *et al.*, 1988). The area of remaining cortex in each section was measured using Neurolucida (MicroBringtField, Colchested, VT) perimeter tracing software. Volume was calculated as the product of the total area (summed over all sections) and the distance between section planes.

2.3.8 Estimation of neuronal density and synaptic density

2.3.8.1 Neuronal Density Measures

The density of neurons in layer V of the perilesion cortex was estimated using the physical disector method (Gundersen *et al.*, 1988). Disector pairs used for neuronal density measures consisted of digital images taken from every other serial semithin section using a Nikon Optiphot-2 light microscope equipped with a rotating stage. The sampling strategy was intended to optimize the consistency of the sampling relative to both the lesion boundary and cortical subregions. Cytoarchitectonics in adjacent 50 μm

thick sections and low magnification semithin images were used to localize the sample area to two subregions within layer V of the agranular cortex medial to the lesion and approximately 1.2-1.6 mm anterior to bregma. One subregion (named "Perilesion") was near the medial boundary of the lesion (as shown in Figure 2.4), excluding fibrotic and necrotic tissue. The second subregion (named "Border") was at least 700 μm medial from the first subregion and was located in the medial agranular cortex near the border of the cingulate cortex. Images of 44,000 μm2 layer V samples were taken using a high-resolution digital camera (DVC Co., Austin, TX) at a final magnification of 830X. Once the images from section 1 were captured, the same sample fields were found and captured in each of the next four sections of the series. The same sampling strategy was applied to another set of 5 serial sections, so that a total of 20 images (10 images for each one of the two subregrions) were captured for each animal. Images from each adjacent set of sections were used as a disector pair and the neurons were counted if they were present in the "reference" section but not present in the "look-up" section (Gundersen *et al.*, 1988). All samples of each set of 5 serial sections were used as both a reference and look-up section so that 16 disector pairs per subregion were used per animal. Unbiased sample frames were placed onto each image in Adobe Photoshop and neurons were identified by multiple criteria including the presence of a nucleus surrounded by cytoplasm and, frequently, the presence of a pyramidal shaped soma. The coefficient of errors (CEs; West and Gundersen, 1990) of the neuronal density estimates per rat ranged from 0.030 to 0.077 (median = 0.058) and mean CEs were similar between groups: 0.056 (LesRehab) to 0.061 (LesCTL). Neuronal density for each of two subregions were calculated by the formula: $Nv = \Sigma Q- / \Sigma v(frame)$, where: $\Sigma Q-$ is the sum of neurons counted per brain and $\Sigma v(frame)$ is the sum of the sample volume (2,252,800 μm3). The sum of the sample volume was calculated as the product of the area of one

sample frame, the distance between section planes (1.6 μm) and the number of samples (16).

2.3.8.2 Synaptic measures

The densities of synapses in layer V, medial to the rostral part of the lesion, in the residual motor cortex were also estimated using the physical disector method. Four sets of four serial adjacent electron micrographic samples (final magnification of 14,000 X), spanning at least 11 sections, were imaged from serial silver gray (70nm) sections. The first set of four serial images was taken in the lateral extent of the Perilesion region sampled for neuronal density estimates. Moving medially, the first section of the next series was taken in the same section as the last section from series 1 and then from 3 additional sections. This was then repeated for the next 2 series. All four series were confined to the Perilesion sampling area for neuronal density. This strategy minimizes the contribution of section-to-section variability in thickness. Occasionally, the presence of artifacts (folds, lead precipitant) required adjustment in the overlap of the series. The first sample of each series was positioned randomly with the exception that cell bodies, capillaries and large dendritic shafts (*e.g.*, the proximal apical shaft) were dodged. At each sample site, four 38 μm^2 digital images were taken with a Hamamatsu 1394 digital camera installed in a Philips 208 transmission electron microscope and then photomerged into a single digital electron micrograph in Adobe Photoshop. The axodendritic synapses were identified by the presence of a post-synaptic density and at least three vesicles in the presynaptic bouton. Each micrograph was used as both a reference and a look-up section for counting the post-synaptic densities (see Figure 4.2C) so that there were 24 disector pairs per brain. The CEs of the synaptic density estimates per rat

ranged from 0.025 to 0.060 (median = 0.043) and group means ranged from 0.041 (LesCTL) to 0.043 (ShamCTL). Synaptic density was calculated by the formula: $Nv = \sum Q^- / \sum v_{(frame)}$, where $\sum Q^-$ is the sum of synapses counted per brain and $\sum v_{(frame)}$ is the sum of the sample volume (188.05 μm^3). $\sum v_{(frame)}$ was calculated as the product of the area of one sample frame (112 μm^2), distance between section planes (70nm), and the number of samples (24). Synapse per neuron in the perilesion area was reported using the value of synaptic density divided by neuronal density of the Perilesion subregion.

2.3.8.3 Perforated synapses and synapses formed by multisynaptic boutons

Perforated (Perf) synapses and synapses formed by multisynaptic boutons (MSBs) were also estimated because these synapse subtypes have been linked with increases in synaptic efficacy (*e.g.*, Geinisman *et al.*, 1991; Geinisman *et al.*, 2001) and have previously been found to be increased in the motor cortex in association with the acquisition of motor skills (Kleim *et al.*, 1998; Jones *et al.*, 1999). MSB and Perf synapses were counted using the physical disector method as described above. Multiple short section series (4 sections/series) were used in this study to provide a greater breadth of sampling; however, this technique limits the ability to reconstruct boutons in three dimensions (which requires much longer section series) and greatly underestimates MSB and Perf synapse density (Jones *et al.*, 1997; Jones, 1999). It has previously been found that the measurement of MSBs and perforated synapses in short series of sections is as sensitive to group differences in the prevalence of these synapse subtypes as reconstruction methods (Jones *et al.*, 1997; Jones, 1999).

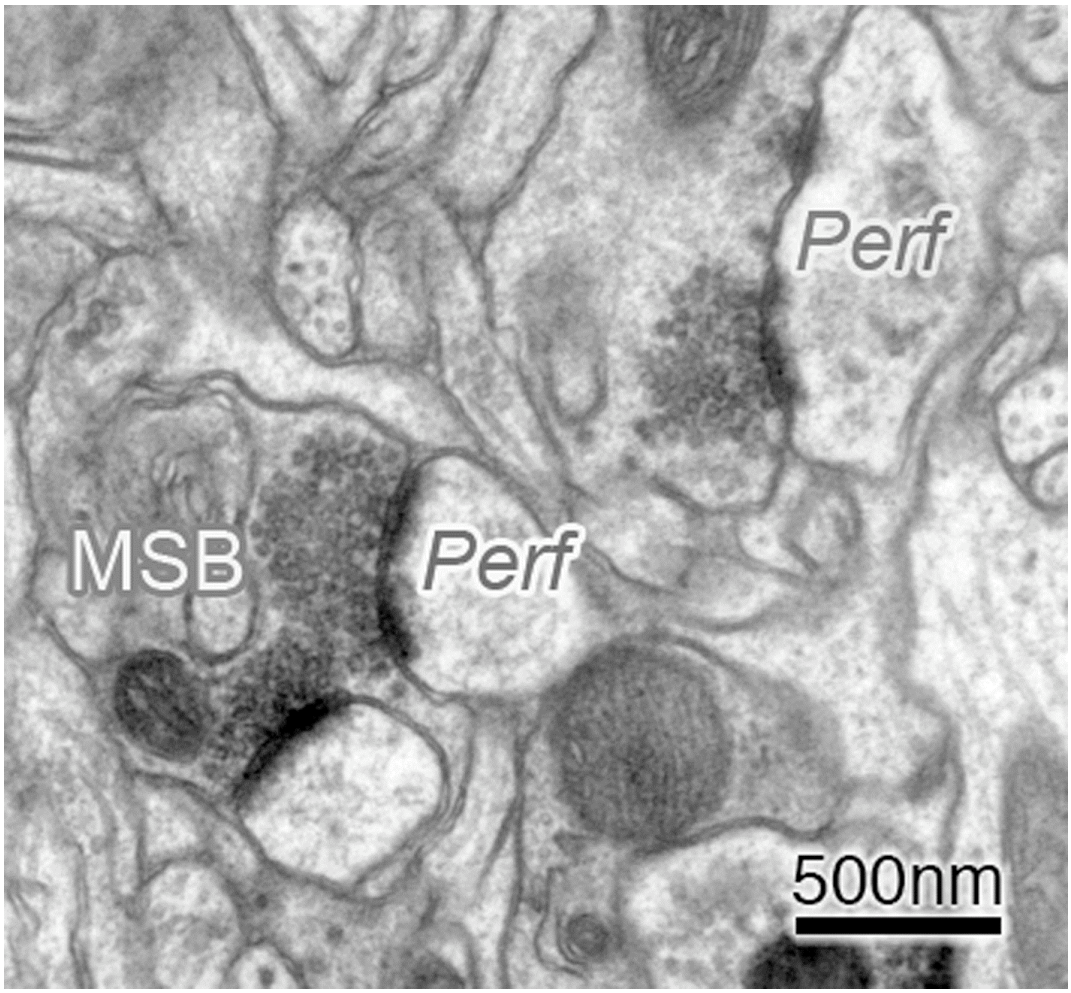

Figure 2.5 Representative electron micrographs of subtypes of layer V motor cortical axodendritic synapses.

Synapses with perforated or segmented post-synaptic densities were identified as perforated (Perf) synapses. Boutons forming synaptic contacts with more than one distinct dendritic element (spine or shaft) were identified as multisynaptic boutons (MSBs). These synapse subtypes were chosen for analysis because they have been linked to increased synaptic efficacy.

2.3.9 Statistical analysis

SPSS (SPSS, Inc.) repeated-measures analyses of variance (ANOVAs) for the effects of Group, Time, and Group by Time interaction were used to analyze the behavioral measures. One-way ANOVAs were used for post hoc analyses when needed in order to test: (i) whether rats that received rehabilitation after unilateral SMC lesions were significantly different from rats that received no rehabilitation control procedures (LesRehab vs LesCTL), and (ii) whether rats with lesions were significantly different from rehabilitation-condition-matched intact controls (LesRehab vs ShamRehab and LesCTL vs ShamCTL). Anatomical data were analyzed using one-way ANOVA with Group (LesRehab, LesCTL, ShamRehab, and ShamCTL) as a factor. Fisher's LSD post hoc analyses were used when needed to futher analyze the difference between groups. Bivariate correlations were used to assess the relationships between the synaptic density and reaching performance in the last two probe tests (averaged), and between synaptic density and abnormal observations in reaching movements at Post-Rehab time points in all lesion animals (LesRehab and LesCTL combined).

2.4 RESULTS

2.4.1 Lesion size and extent were similar between the two lesion groups.

Reconstructions of the lesions indicated similarity in the range and extent of the lesions between groups. All lesions in this study were found to produce damage to the SMC and the damage was similar between the two lesion groups (Figure 2.6). Measurements of the volumes of the remaining SMC and contralateral hemisphere indicated that lesion sizes were similar in the two lesion groups. The remaining volume of lesion cortex in mm^3 (mean ± SEM) was 100.72 ± 1.33 in the LesRehab group and 99.57 ± 1.00 in the LesionCTL group, and the volume of contralateral SMC was 108.30 ± 2.00 in the LesRehab group and 106.02 ± 1.22 in the LesionCTL group.

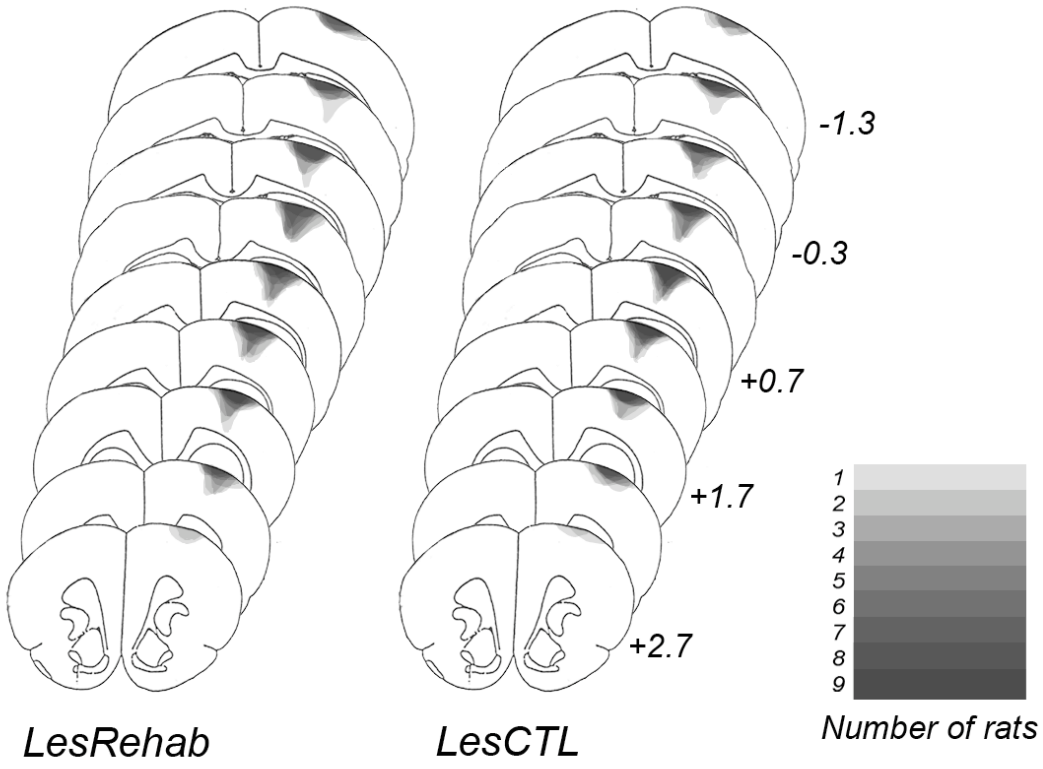

Figure 2.6 Reconstruction of the extent and placement of focal unilateral SMC lesions in LesRehab and LesCTL groups.

Reconstructions of each lesion are overlaid onto the left hemisphere of schematic coronal sections so that the darkest areas have the greatest extent of lesion overlap between subjects. Numbers to the right are approximate coordinates (mm) relative to bregma.

2.4.2 Skilled reaching as rehabilitation improves reaching success in rats after unilateral ischemic lesions.

Figure 2.7A shows the percentage of successful retrievals with the impaired forelimb on the single pellet skilled reaching task. The data include the last training day before surgeries (PreOP) and 5 probe test days (Probe 1-5, one week apart) over 4 weeks. Analysis using repeated measures ANOVA with Group (LesRehab, LesCTL, ShamRehab, and ShamCTL) and Time as factors revealed significant Group ($F(3,30)=4.95$, $p<0.01$), Time ($F(5,150)=17.59$, $p<0.001$), and Group by Time interaction effects ($F(15, 150)=1.83$, $p<0.05$). In post hoc analysis, lesion animals with rehabilitation had greatly enhanced reaching performance with the impaired forelimb compared to no rehabilitation controls on Probe tests 4 and 5 (p's<0.05). Without rehabilitation, lesion animals showed enduring impairments of reaching performance on Probe 1, 2, 4, and 5 (p's<0.05, $p=0.056$ in Probe 3) compared with sham animals. In contrast, lesion animals with 4 weeks of rehabilitation only showed significantly worse reaching performance on Probe 1 ($p<0.05$) compared to sham animals.

Figure 2.7B shows five rehabilitation training days per week over four weeks for the two rehabilitation groups. A repeated measures ANOVA revealed both a significant Group ($F(1,15)=4.69$, $p<0.05$), Time ($F(19, 285)=14.49$, $p<0.001$) and Group by Time interaction effect ($F(19, 285)=4.96$, $p<0.001$). Post hoc analyses indicated that the Les Rehab group was significantly different from the Sham Rehab group from the beginning of rehabilitative training up to and including the first training day in the second week.

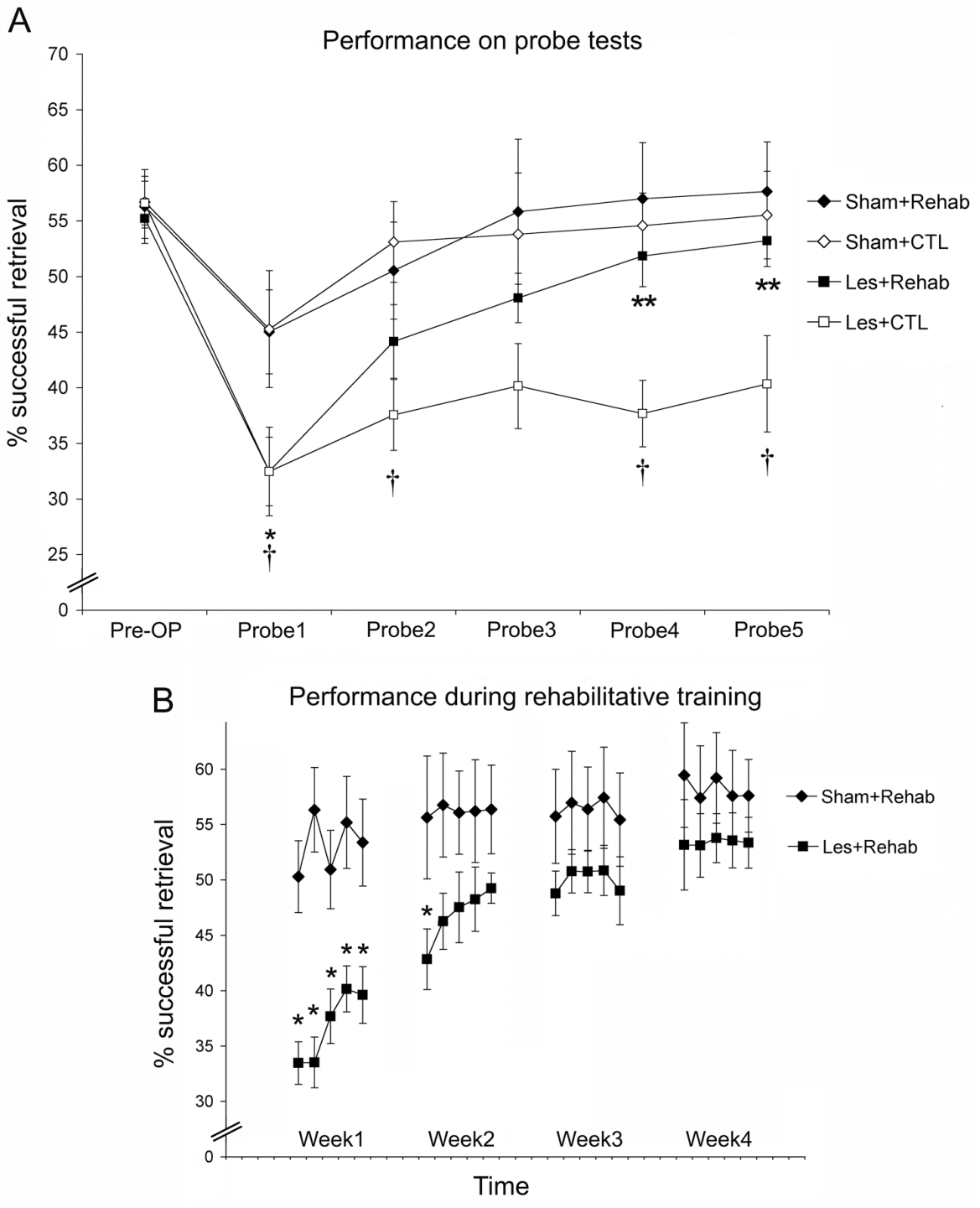
A
Performance on probe tests
% successful retrieval
70
65
60
55
50
45
40
35
30
25
0
Pre-OP
Probe1
Probe2
Probe3
Probe4
Probe5
Sham+Rehab
Sham+CTL
Les+Rehab
Les+CTL
B
Performance during rehabilitative training
% successful retrieval
60
55
50
45
40
35
30
0
Week1
Week2
Week3
Week4
Time
Sham+Rehab
Les+Rehab

Figure 2.7 Skilled reaching as rehabilitation improves reaching success in rats after unilateral ischemic lesion.

The percentages of successful retrievals on weekly probe tests of skilled reaching performance and during rehabilitative training are shown. (A) The two lesion groups had severe impairments in skilled reaching performance after surgeries. Lesion animals receiving rehabilitation (LesRehab) had better reaching performance on probes 4 and 5 compared to no rehabilitation controls (LesCTL). Without rehabilitation, lesion animals showed enduring impairments in reaching compared to sham animals (ShamCTL). In contrast, lesion animals with 4 weeks rehabilitation only showed significantly worse reaching performance on Probe 1 compared to sham animals (ShamRehab). (B) Lesion animals showed improvements in reaching performance over the course of rehabilitative training. Data are means ± SEM. *$p<0.05$ significantly different from rehabilitation matched sham animals. **$p<0.05$ significantly different from non-rehabilitation matched lesion animals. †$p<0.05$ significantly different from non-rehabilitation matched sham animals.

2.4.3 Skilled reaching as rehabilitation normalizes some categories in reaching movements.

After unilateral SMC lesions, both lesion groups had an increase in the number of movement abnormalities while performing the single pellet retrieval task, especially in the last four categories (Grasp, Supination I, Supination II, and Release) of the reaching sequence. Repeated measures ANOVA showed a Group by Time interaction effect in both the Grasp ($F(6,60)=3.72$, $p<0.01$) and Supination II ($F(6,60)=6.75$, $p<0.001$) categories. Figure 2.8A shows the number of abnormal grasping movements. Post hoc analyses indicated that the number of abnormal movements between lesion and rehabilitation-matched sham groups were not different after surgery (Rehab: $p=0.15$, CTL: $p=0.052$), though the differences between LesCTL and LesSham were approaching significance. After rehabilitation, the LesCTL group had a greater number of abnormal movements compared to LesRehab ($p<0.001$) and ShamCTL ($p<0.01$) groups.

Figure 2.8B shows observations of abnormal movement in the Supination II category. LesRehab had a greater number of abnormal movements compared to rehabilitation-matched sham animals following surgeries ($p<0.05$), but not after rehabilitation. Instead, the LesRehab group had significantly less abnormalities compared to LesCTL after rehabilitation ($p<0.01$). Without rehabilitation, the LesCTL group had a greater number of abnormal movements compared to no-rehabilitation-matched sham animals at both Post-OP ($p<0.01$)and Post-Rehab ($p<0.05$) time points.

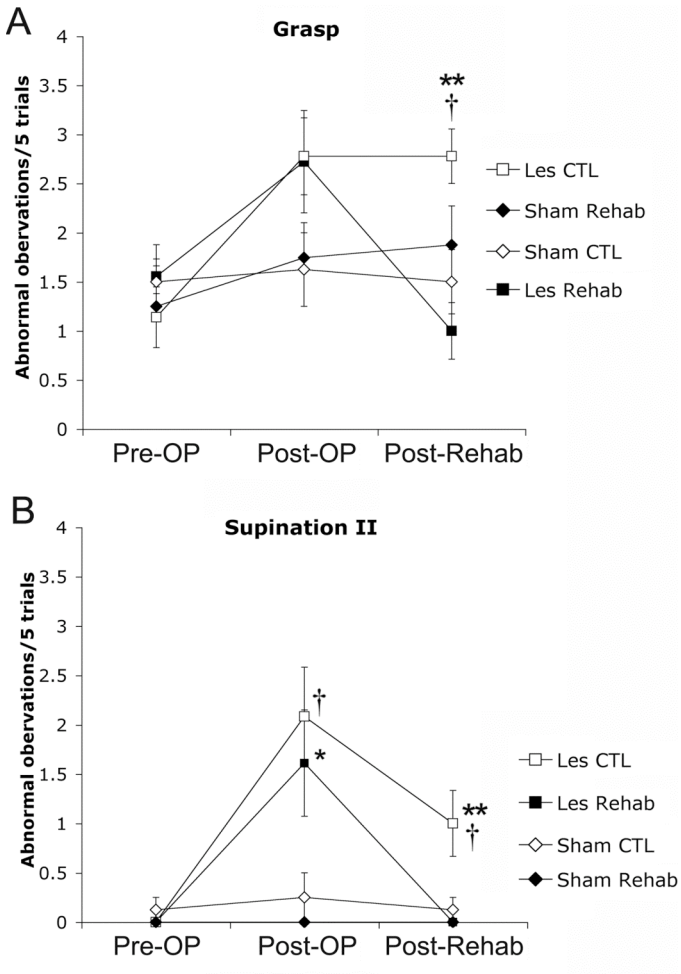

Figure 2.8 Skilled reaching as rehabilitation normalizes some categories in reaching movement.

Observations of abnormalities (from five trials) found in the Grasp and Supination II categories of reaching movements are shown. (A) The LesCTL group had a greater number of abnormal grasping movements compared to the LesRehab and ShamCTL groups after rehabilitation. (B) In the Supination II category, the LesRehab group had a greater number of abnormal movements compared to ShamRehab following surgeries, but not after rehabilitation. Instead, the LesRehab group had significantly less abnormalities compared to the LesCTL after rehabilitation. The LesCTL group had a greater number of abnormal movements compared to ShamCTL at both Post-OP and Post-Rehab time points. Data are means ± SEM. *$p<0.05$ significantly different from rehabilitation-matched sham animals. **$p<0.01$ significantly different from lesion animals that received rehabilitation. †$p<0.05$ significantly different from non-rehabilitation matched sham animals.

2.4.4 Impairments in coordinated forelimb usage during locomotion recovered regardless of rehabilitation condition.

Figure 2.9 shows the percentage of errors (foot slips) made per step with the contralesional/impaired forelimb one day before surgery, three days after surgery, and one day after rehabilitation. In repeated measures ANOVA with Group and Time as factors, there were significant Group ($F(3,30)=5.52$, $p<0.01$), Time ($F(2,60)=32.35$, $p<0.001$), and Group by Time interaction effects ($F(6,60)=7.92$, $p<0.001$). Post hoc analysis showed that the animals in the two lesion groups made significantly more errors with their impaired forelimb compared with rehabilitation-matched sham animals three days after surgery (p's<0.001); these effects were gone after the rehabilitation period in both lesion groups.

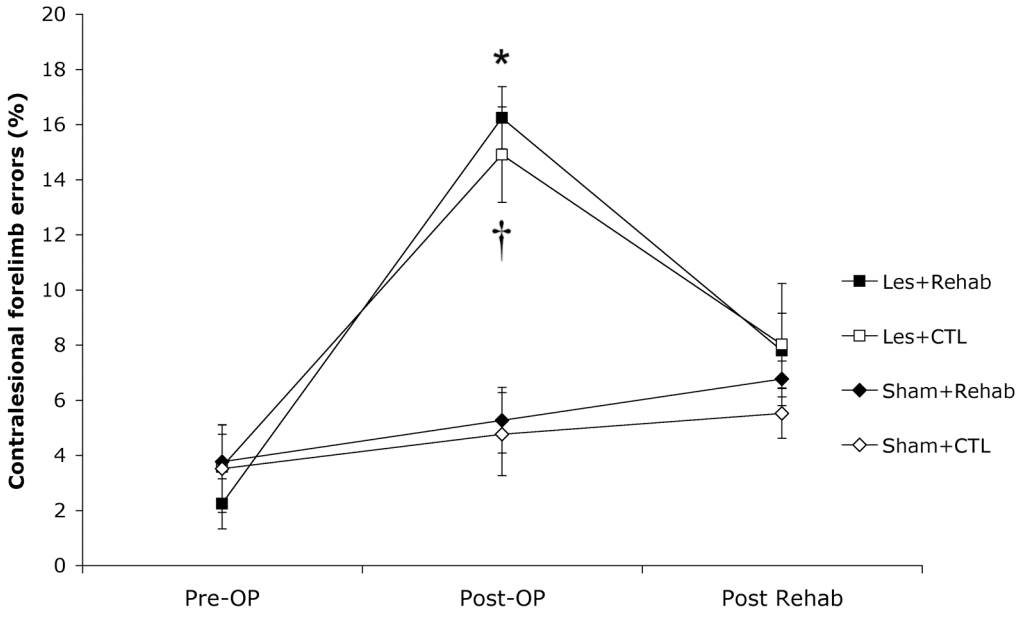

Figure 2.9 Impairments in coordinated forelimb usage during locomotion recovered regardless of rehabilitation condition.

The percentage of errors (foot slips) made per step with the contralesional/impaired forelimb is shown. Animals in the two lesion groups made significantly more errors with the contralesional forelimb compared with rehabilitation-matched sham animals three days after surgery. All lesion animals had recovered to control levels by the post-rehabilitation test date such that it would not be possible to detect a further improvement as a result of the motor rehabilitation. Data are means ± SEM. *$p<0.001$ significantly different from rehabilitation-matched sham animals. †$p<0.001$ significantly different from non-rehabilitation matched sham animals.

2.4.5 Rehabilitation in lesion animals increases synaptic density in layer V of the perilesion cortex.

Figure 2.10 shows the axodendritic synaptic density measured in the motor cortex medial to the lesion. Synaptic density was significantly different between groups ($F(3,30)=6.47$, $p<0.01$). Fisher's LSD post hoc analyses showed that lesion animals receiving rehabilitation had significantly greater synaptic density in layer V of the perilesion cortex compared to no rehabilitation animals and rehabilitation-matched sham controls. Without rehabilitation, there were no significant differences between the lesion and sham animals. In the measure of neuronal density (Table 2.1), there were no significant differences between any groups in either the perilesion or border subregions (p's >0.05).

Table 2.1 Neuronal density in layer V of perilesion cortex

Group	Perilesion	Border
Les+Rehab (n=9)	31.18 ± 3.09	35.50 ± 2.57
Les+CTL (n=9)	33.11 ± 0.86	35.87 ± 2.45
Sham+Rehab (n=8)	32.07 ± 1.16	32.80 ± 1.48
Sham+CTL (n=8)	33.11 ± 2.55	31.97 ± 1.82

Data are means ± SEM (10^3 neurons per mm^3)

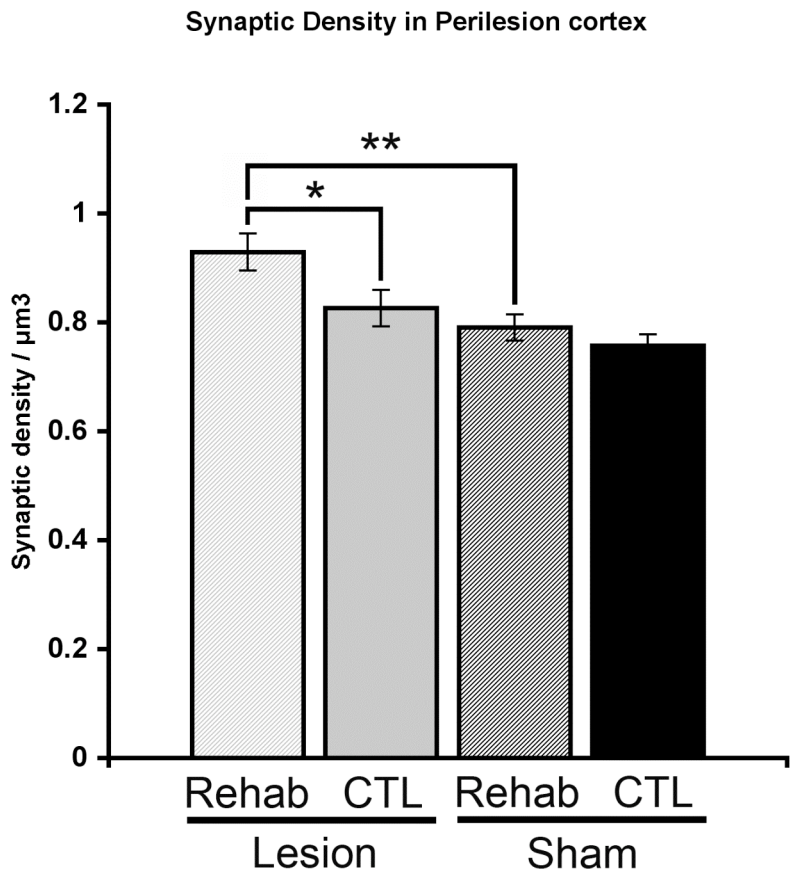

Figure 2.10 Rehabilitation in lesion animals increases synaptic density in layer V of the perilesion cortex.

Lesion animals that received rehabilitation had significantly greater synaptic density in layer V of the perilesion cortex compared to no rehabilitation animals (*$p<0.05$) and rehabilitation-matched sham controls (**$p<0.01$). Data are means ± SEM.

2.4.6 Rehabilitation in lesion animals increases efficacious synapse subtypes in layer V of the perilesion cortex.

Figure 2.11 shows the density of synapses with perforated post-synaptic densities (Perf) and synapses formed by multisynaptic boutons (MSBs). Densities of both perforated synapses and MSBs were significantly different between groups (Perf: $F(3,30)=3.05$, $p<0.05$; MSB: $F(3,30)=4.51$, $p<0.01$). Consistent with the findings of total axodendritic synaptic density, Fisher's LSD post hoc analyses also showed that lesion animals receiving rehabilitation had significantly greater synaptic densities of Perf and MSB in layer V of the perilesion cortex compared to no rehabilitation animals and rehabilitation-matched sham controls. Without rehabilitation, there were no significant differences in both perforated and MSB synaptic densities between lesion and sham animals (p's>0.05).

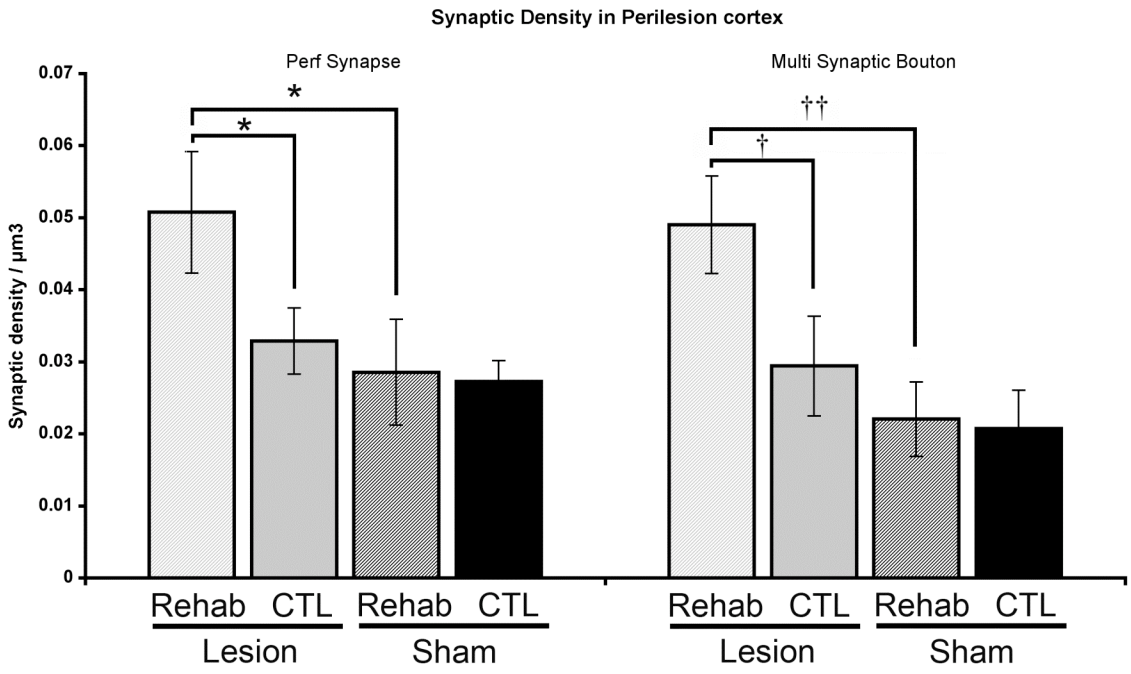

Figure 2.11 Rehabilitation in lesion animals increases efficacious synapse subtypes in layer V of the perilesion cortex.

Lesion animals that received rehabilitation had greater perforated synaptic density (*p<0.05) and MSB density (†p<0.05) in layer V of the perilesion cortex compared to no rehabilitation animals. And these aniamls also had greater perforated synaptic density (*p<0.05) and MSB density (††p<0.01) in layer V of the perilesion cortex compared to rehabilitation-matched sham controls. Data are means ± SEM.

2.4.7 The density of perforated synapses is positively correlated with functional outcome.

Figure 2.12 illustrates that the density of perforated synapses was significantly correlated with averaged reaching performance (% successful retrievals) in the last two probe tests in all lesion animals ($p<0.05$, $r= 0.54$). The correlation between synaptic density and averaged reaching performance across the last two probes approached significance ($p=0.085$, $r=0.42$).

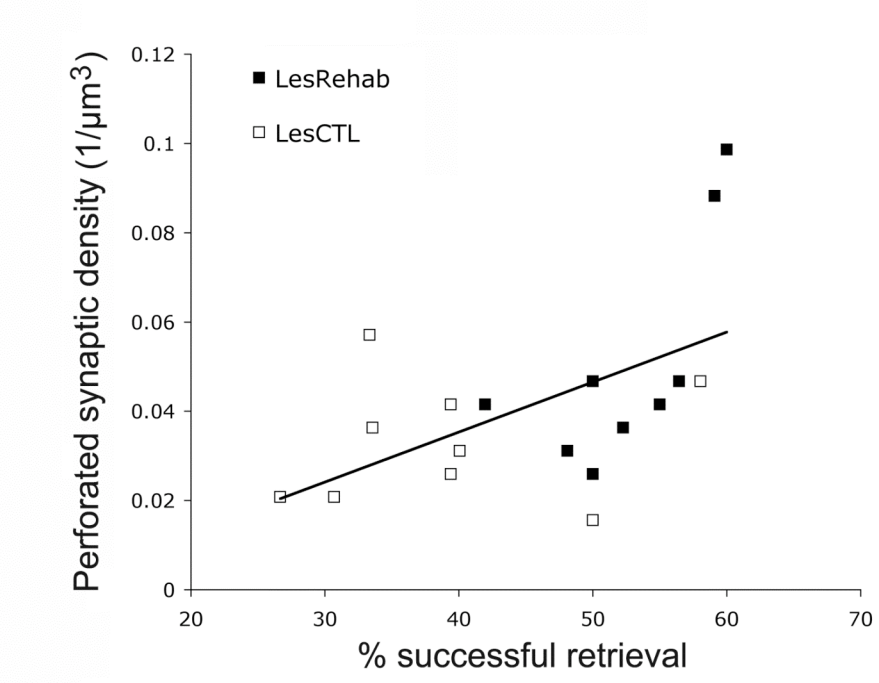

Figure 2.12 The density of perforated synapses is correlated with reaching success on the single pellet retrieval task.

The density of perforated synapses was significantly correlated with averaged reaching performance (% successful retrievals) in the last two probe tests in all lesion animals ($p<0.05$, $r = 0.54$).

2.4.8 The density of synapses formed by multisynaptic boutons is negatively correlated with the number of abnormal grasping movments after rehabilitation.

Figure 2.13 illustrates that the density of MSBs was negatively correlated with the number of abnormal grasping movments in all lesion animals (LesRehab and LesCTL combined) at Post-Rehab time point ($p<0.05$, $r= - 0.54$).

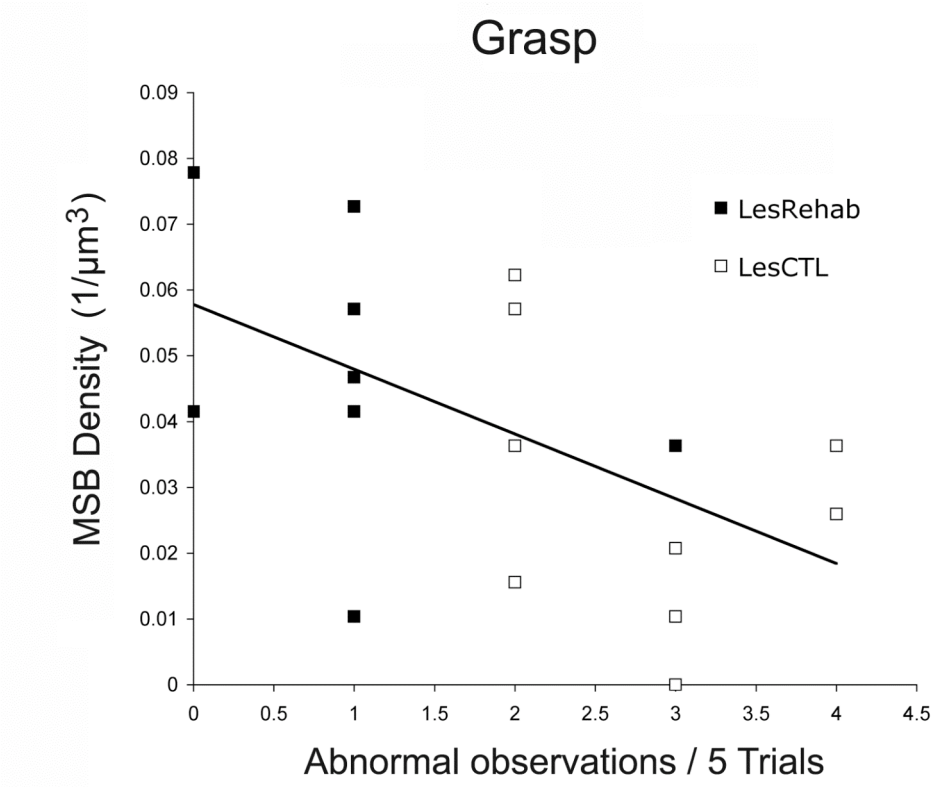

Figure 2.13 The MSB density is negatively correlated with abnormal grasping movements.

The MSB density was negatively correlated with the number of abnormal grasping movements in all lesion animals at Post-Rehab time point ($p<0.05$, $r= -0.54$).

2.5 DISCUSSION

Motor rehabilitative training improved reaching success in the single pellet retrieval task and reduced lesion-induced abnormal reaching movements in rats with unilateral ischemic SMC lesions compared to lesion control animals that received no rehabilitation. Rehabilitation in lesion animals was linked with an increased density of layer V synapses in the perilesion cortex medial to the rostral part of the sensorimotor (SMC) infarct. It was also found that rehabilitation in lesion animals increased the density of synapses formed by multisynaptic boutons (MSBs) and synapses with perforated post-synaptic densities compared to both lesion rats without rehabilitation and rehabilitation-matched sham rats. Layer V perforated synaptic density was positively correlated with functional outcome in animals with unilateral ischemic lesions, whereas layer V MSB synaptic density was negatively correlated with abnormal grasping movements after rehabilitation in these same rats.

2.5.1 Motor rehabilitation improves reaching performance in the impaired forelimb.

In this study, motor rehabilitation using a skilled reaching task, the single pellet retrieval task, enhanced recovery of reaching performance by increasing the number of pellets successfully retrieved and by reducing abnormal grasping movements. This finding is consistent with previous findings that rehabilitative training significantly contributes to functional recovery following focal brain injury in rats (*e.g.* Biernaskie and Corbett, 2001; Conner *et al.*, 2005). A recent study by Gharbawie *et al.* (2006) showed that the digit flexion and closing used for grasping in skilled reaching is abolished by focal ischemic lesions. The present results extend these previous findings by indicating that

rehabilitation using skilled reaching normalizes grasping and further supinating movements in lesion animals. However, this intensive task-specific rehabilitation had no effect on coordinated forelimb use during locomotion. Both lesion groups were indistinguishable from sham animals for foot-slips during locomotion approximately four weeks after lesions. These findings indicate that spontaneous recovery did occur on certain behavioral tests in rats which did not receive rehabilitation, while not occurring on certain specific tasks in the skilled reaching test.

2.5.2 Motor rehabilitation facilitates synaptic structural changes which are correlated with functional outcome in lesion rats

After rehabilitative training, animals with unilateral ischemic SMC lesions had significantly greater densities of total synapses along with efficacious synapse subtypes in layer V of perilesion cortex compared to no rehabilitation controls. Additionally, the perforated synaptic densitiy in layer V was significantly correlated with reaching success in animals with unilateral ischemic lesions, while the total synaptic density approached significance. MSB synaptic density in layer V was also found to be negatively correlated with abnormal grasping movements after rehabilitation in lesion rats. It is well established that the acquisition of a skilled reaching task is mediated by motor cortical plasticity in intact animals (Monfils *et al.*, 2005; Adkins *et al.*, 2006a). Skilled reach training increases the complexity of dendritic processes (Greenough *et al.*, 1985; Withers and Greenough, 1989) and synapses per neuron (Kleim *et al.*, 2002; Kleim *et al.*, 2004) in the motor cortex opposite the trained limb. Learning-induced synaptogenesis includes increases in perforated synapses and synapses formed by multisynaptic boutons

(Jones *et al.*, 1999) and synaptogenesis is co-localized with expanded caudal forelimb motor maps (Kleim *et al.*, 2002). Synaptic plasticity in remaining cortex is also likely to be an important mediator of recovery from brain injury (Kolb, 2003; Nudo, 2003). Cortical ischemic injury results in cascades of growth inhibitory and growth permissive cellular and molecular changes in remaining cortex (Carmichael, 2006) which are likely to be sensitive to the effects of behavioral experience. Subtotal motor cortical lesions disrupt the functional integrity of remaining motor cortical areas (Nudo and Milliken, 1996). However, practice in skilled reaching improves post-lesion reaching deficits and reinstates motor maps (Nudo *et al.*, 1996; Friel *et al.*, 2000; Conner *et al.*, 2005; Ramanathan *et al.*, 2006). Disruption of motor cortical plasticity decreases reaching performance (Kleim *et al.*, 2003a). In rats, injection of protein synthesis inhibitors within the motor cortex causes synapses to be lost, motor maps to disappear and reaching behavior to become impaired (Kleim *et al.*, 2003a). Together, these findings suggest that plasticity in the perilesion cortex is likely to be an important contributor to the recovery of motor skill.

The present study also found that the enhancement of synaptic structural changes after rehabilitative training was not found in the area adjacent to the SMC in sham animals. Kleim *et al.*, (2002, 2004) found that skilled reach training increases synaptic structural plasticity in the motor cortex and that synaptogenesis is co-localized with expanded caudal forelimb motor maps opposite the trained limb. The present results extend the previous findings by indicating that the synaptic plasticity induced by skilled reach training in intact animals does not include the adjacent area sampled in this study that was found to be reorganized in lesion animals.

2.5.3 Implications for rehabilitation in brain damage recovery

Reorganization of motor representations has been linked with functional recovery after various types of brain injury (Green, 2003; Jang *et al.*, 2002) and ablation of the reorganized cortex reinstates functional deficits (Castro-Alamancos and Borrel, 1995; Conner *et al.*, 2005). Expanded rostral forelimb motor maps were found in the perilesion cortex of rats with focal cortical injury that received rehabilitation compared to lesion control animals that received no rehabilitation, and lesion rats that received no rehabilitation did not show expanded rostral forelimb maps compared to intact rats (Conner *et al.*, 2005). The present results extend these previous findings by suggesting that synaptic plasticity may mediate the reinstatement of the neural integrity and reorganization of circuitry that underlies functional improvements. Conner *et al.* (2005) suggested that functional recovery following cortical injury requires the reorganization of motor representations which is dependent upon the basal forebrain cholinergic system. An increased dendritic density in layer V of the cortex lateral to the lesion was found in rats with focal cortical lesions that received electrical cortical stimulation during rehabilitation (Adkins-Muir and Jones, 2003). Kolb *et al.* (1997) found dendritic atrophy in the remaining motor cortex after unilateral devascularizing lesions in the sensorimotor cortex. Further research is needed to understand how this motor cortical plasticity may be coordinated with changes in connected cortical and subcortical regions.

Chapter 3: Protein synthesis inhibition in the perilesion cortex disrupts functional recovery induced by rehabilitative training after a unilateral cortical infarct in rats

3.1 ABSTRACT

The previous study showed that rehabilitative skilled reaching with the impaired forelimb enhances functional recovery and the structural reorganization of the perilesion cortex after unilateral focal ischemic lesions in the SMC. Whether the synaptic structural plasticity seen in the perilesion cortex is related to the animals' behavioral improvements requires further investigation. Kleim *et al.* (2003a) found that protein synthesis inhibition (PSI) by anisomycin injection in the SMC not only impairs skilled movement but also causes a long term loss of motor representations and decreases synapse number and size in the SMC. The purpose of this study was to determine whether PSI in the perilesion cortex, where structural reorganization takes place following an ipsilateral SMC ischemic lesion, disrupts the *recovered* reaching performance induced by rehabilitative training. Rats proficient in skilled reaching with one limb received unilateral ischemic (endothelin-1 induced) lesions of the contralateral (to the reaching limb) sensorimotor cortex along with cannula-implantation. After rehabilitative training to a plateau, anisomycin (100 μg/μl in 1.0 μl ACSF) was injected into the perilesion cortex while two control groups received either vehicle injections (1.0 μl ACSF) in the perilesion cortex or anisomycin injections in the ipsilesional parietal cortex. The results indicated that PSI in the perilesion cortex impedes the recovered skilled reaching performance seen in

rehabilitated lesion rats. This indicates that the reorganized cortex is likely to be an important contributor to behavioral recovery.

3.2 INTRODUCTION

Focal cortical lesions in rats (Whishaw, 2000), monkeys (Nudo and Milliken, 1996), and humans (Green, 2003) result in impairments in skilled reaching performance. With subsequent rehabilitative training in skilled reaching, the reorganization of motor representations is induced and is thought to contribute to relevant functional recovery in rats (Castro-Alamancos and Borrell, 1995), monkeys (Friel *et al.*, 2000; Nudo *et al.*, 1996b), and humans (Leipert *et al.*, 2000; Green 2003). Ablation of the reorganized cortex in rats (Castro-Alamancos and Borrel, 1995; Conner *et al.*, 2005), injection of a GABA agonist (muscimol) into the adjacent cortex in rats (Hernandez and Schallert, 1990), or inactivation of the perilesion cortex by disruptive transcranial magnetic stimulation (TMS) in humans (Fridman *et al.*, 2004) reinstates the functional deficit. These studies suggest that functional reorganization in the perilesion cortex after rehabilitative training plays an important role in functional recovery of the impaired limb. However, these previous studies focused on the electrophysiological mapping of motor cortical representations.

According to the findings in Chapter 2, rehabilitative skilled reaching with the impaired forelimb can enhance functional recovery and the structural reorganization of the perilesion cortex in rats with focal motor cortical lesions. However, whether the structural integrity of the reorganized cortex is required for the maintenance of rehabilitation-induced behavioral improvements remains unknown. Kleim *et al.* (2003a) showed that the structural integrity within the motor cortex of intact rats can be changed by focal protein synthesis inhibition. Anisomycin has been found to block

newly synthesized protein in various paradigms. For example, Davis and Squire (1984) showed that PSI during or shortly after training inhibits the formation of long-term memories. Focal anisomycin injections into the hippocampus have been shown to result in the inhibition of spatial learning and memory (*e.g.*, Naghdi *et al.*, 2003; Morris *et al.*, 2006), whereas injections into the nucleus accumbens have been shown to block the early consolidation of instrumental learning (Hernandez *et al.*, 2002) in rats. Unlike the more traditional method used to determine the effect of protein synthesis inhibition (PSI) on the engram, in which anisomycin is injected during or shortly after daily training or testing, in the present study anisomycin injections were performed at the end of experiments, once structural reorganization in the perilesion cortex had already taken place. The injections were thus used to disrupt synaptic connectivity in the newly reorganized perilesion cortex, an effect of the injections suggested by Kleim *et al.* (2003a) in their findings that protein synthesis inhibition (PSI) by anisomycin injection in the SMC not only impairs skilled movement but also causes a long term loss of the motor representation map and decreases synapse number and size. However, Luft *et al.* (2004) showed that PSI by anisomycin injection in the motor cortex impairs motor skill learning but does not disrupt learned motor performance. In this study, two experiments were conducted to address the effects of PSI in the SMC in intact rats and the effects of PSI in the perilesion cortex in rats with unilateral ischemic SMC lesions.

The purpose of Experiment 1 was twofold: (1) to verify whether PSI in the SMC disrupts learned reaching performance in intact rats and (2) to determine whether PSI in the SMC causes a loss of the forelimb movement representations revealed by intracortical

microstimulation (ICMS). A group of intact rats were trained to a plateau on a skilled reaching task with their preferred forelimbs and then received cannula-implantation surgeries that targeted either the forelimb representation region of the SMC or the region adjacent to the SMC contralateral to the preferred forelimb. Anisomycin was then injected into the SMC or the adjacent area prior to performance of the skilled reaching task in order to test for the effects of PSI on learned reaching performance. All animals were perfused one day after the last probe test. Another group of rats received intracortical microstimulation (ICMS) surgeries to test the effects of PSI on forelimb movement representation in the motor cortex. In Experiment 2, the purpose was to determine whether anisomycin injection in the perilesion cortex would disrupt the regained reaching performance that occurred following motor-rehabilitation in rats with unilateral ischemic SMC lesions. Before surgeries, all rats were trained to a plateau on a skilled reaching task with their preferred forelimbs. Unilateral focal ischemic lesions were then made in the forelimb representation region of the sensorimotor cortex (SMC) contralateral to the preferred forelimb along with a cannula implantation that targeted either the region adjacent to the SMC (perilesion cortex) or the parietal cortex. Skilled reach training was used as a rehabilitative training task and probe trial tests were held once a week over a 3 week span. After rehabilitation, anisomycin was injected into the perilesion cortex while controls received either anisomycin in the parietal cortex or vehicle in the perilesion cortex. Performances in both experiments were evaluated by determining the animals' percentages of successful retrievals using their impaired/preferred forelimbs. Layer II/III and V of the motor cortex were assayed for

the ratio of immunoreactivity for synaptophysin, an integral membrane glycoprotein in pre-synaptic vesicles, between two hemispheres.

3.3 MATERIALS AND METHODS

3.3.1 Subjects and experimental designs

A total of fourty-three rats were used in the present studies. In Experiment 1, six and thirteen adult, 3 to 4 month old, male Long-Evans hooded rats were used for testing the effects of anisomycin injections in the SMC on motor representation maps and skilled reaching performance, respectively. In Experiment 2, twenty-four adult male Long-Evans hooded rats were used for testing the effects of anisomycin injections in the perilesion cortex on skilled reaching performance in rats with unilateral ischemic infarcts. The rats were housed in pairs in transparent cages on a 12:12 hour light:dark cycle and received water *ad libitum*. Rats used for behavioral testing were placed on scheduled feeding (15g, once per day) to ensure rats were not sated at the time of testing. All animal use was in accordance with a protocol approved by the Animal Care and Use Committee of the University of Texas at Austin.

In Experiment 1, animals received cannula implantations targeting either the SMC (SMC group, n=7) or the region adjacent to the SMC (ADJ group, n=6) following pre-operative single pellet skilled reach training with the preferred forelimb. Animals in the ADJ group were used as controls to verify whether PSI in the area adjacent to the SMC , where structural reorganization takes place folowing an ipsilateral ischemic lesion in the SMC, results in the disruption of learned skilled reaching in intact animals. Animals were randomly divided into SMC and ADJ groups with the exception that they were matched as closely as possible for pre-operative skilled reaching performance. Four

days after surgeries, all animals' reaching performances were tested for 2 consecutive days to assess the residual effect of cannula-implantation. Then all animals were infused with vehicle (ACSF, 1.0 μl) and tested 2 hours and one day after infusion. Two different volumes of anisomycin, 0.5 and 1.0 μl (100 μg/μl), were infused to measure the dosage required to yield an appropriate disruption of reaching performance, and all animals were tested once a day until the effects of the anisomycin subsided. Animals were perfused intracardially immediately following the last testing session, and the brain tissue was processed for immunocytochemistry study.

In Experiment 2, following pre-operative single pellet skilled reach training with their preferred forelimbs, animals received unilateral ischemic lesions in the forelimb representation region of the SMC contralateral to their preferred forelimbs along with cannula implantations that targeted either the region adjacent to the SMC (perilesion, n=18) or the parietal cortex (n=6). Animals were randomly divided into perilesion and parietal groups with the exception that they were matched as closely as possible for pre-operative skilled reaching performance. Four days after surgeries, all animals received one probe trial, and then four animals (with perilesion cannula implants) were chosen as no rehabilitation controls while the others began rehabilitation for 3 weeks. After rehabilitation, all animals were infused with vehicle and then tested 2 hours and one day later. The perilesion cannula-implantation animals were then randomly divided into either an anisomycin (ANI, n=8) or vehicle injection group (VEH, n=6), with the exception that they were matched as closely as possible for skilled reaching performance, and received injections accordingly. The parietal cannula-implantation animals (Par, n=6) were also used as controls which received anisomycin injections at identical time

points as the perilesion cannula-implantation animals. All animals were tested 1-2 hours after infusion and then were perfused intracardially immediately thereafter. The reach training method used in both experiments was the same as that described in the previous chapter, and the performances were evaluated by determining the animals' percentages of successful retrievals using their impaired/preferred forelimbs.

3.3.2 Surgical procedures

In Experiment 1, animals underwent unilateral cannula implantation surgeries targeting either the SMC or the region adjacent to the SMC contralateral to the preferred forelimb. Rats were anesthetized with a cocktail of ketamine (90 mg/kg) and xylazine (9 mg/kg). The SMC implantations were aimed at the center of the overlapping primary somatosensory and primary motor cortical representation regions of the forelimb (Donoghue and Wise, 1982), whereas the cannula implantations that targeted the region adjacent to the SMC were aimed at the perilesion area discussed in the last chapter that had been found to have more synaptic plasticity after rehabilitation. Injection cannula systems (Plastics One Inc., VA) include the guide cannula, internal cannula (500 μm longer than the guide cannula), and dummy cap (same length as the guide cannula). The external guiding cannulas (457μm in diameter) were implanted through burrholes drilled into either the center of the SMC (1mm anterior, 3mm lateral, depth 700 μm relative to bregma) or in the adjacent region (1.4 mm anterior, 1.5 mm lateral, depth 700 μm). Burrholes and cannulas were then covered with UV secured dental cement and guide cannulas were covered with dummy caps. Figure 3.1 shows the tract of guiding

cannulas implanted in the center of the SMC and the area adjacent to SMC. No cortical damage apart from the tract itself was found.

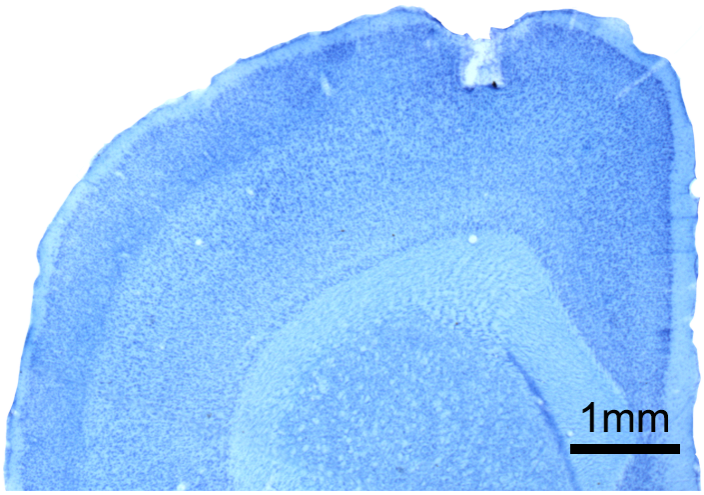

Figure 3.1 Representative tract of guiding cannula implant in the area adjacent to the SMC.

No cortical damage apart from the tract itself was found.

In Experiment 2, animals received unilateral ischemic lesions along with ipsilateral cannula implantation surgeries targeting either the region adjacent to the SMC or the parietal cortex contralateral to the preferred forelimb. After removing the skull and dura between 1.5 mm posterior and 2.5mm anterior to bregma, and between 3.0 and 4.5 mm lateral to midline, lesions were produced by placing 2.5 µl of endothelin-1 (80µM, 0.2µg/µl in sterile saline) directly onto the pial surface. Endothelin-1 was applied in 2 drops (1.5 and 1.0 µl each) spaced 2 min apart and the surgical site was left undisturbed for 10 min after the last drop. The external guiding cannulas were implanted through burrholes drilled into either the perilesion cortex (1.4 mm anterior, 1.5

mm lateral, depth 700 μm, same as the adjacent to the SMC region in Exp1) or the parietal cortex (6mm posterior, 3mm lateral, depth 700 μm relative to bregma). Gelfoam was trimmed and used to fill the craniectomy and then UV secured dental cement was used to cover the gelfoam, burrholes, and cannulas.

3.3.3 Intracortical injections

3.3.3.1 Intracortical injections in intracortical microstimulation mapping

Pulled pipettes (30 to 50 μm in diameter) were used for all injections during intracortical microstimulation surgeries. In accordance with Donoghue and Wise (1982), the injection site was chosen at the center of the SMC (1.0 mm anterior and 3.0mm lateral to bregma) or in the region adjacent to the SMC (1.4 mm anterior and 1.5 mm lateral to bregma). The tip of the pipette was lowered approximately 1500 μm into layer V of the SMC, and the injectate was infused over 1 minute. The pipette was then left undisturbed for 2 minutes to allow for diffusion of the injectate. Either 1.0 μl of 100 μg/μl of anisomycin in ACSF or 1.0 μl of vehicle (ACSF) was injected into the SMC of one hemisphere of an animal while the other hemisphere received the injection of the other injectate. The order of anisomycin and vehicle injection was counterbalanced in six animals and the experimenter who did the mapping was blinded to the injection condition. Injection of anisomycin into the region adjacent to the SMC was made bilaterally in one animal, and was made after vehicle injection into the center of the SMC in two other animals.

3.3.3.2 Intracortical injections using the cannula-implantation system

Animals were briefly sedated with isoflurane (< 2 min) for all of the injections using the cannula implantation system. The injection cannula was advanced through the guiding cannula at the time of injection. Either 1.0 µl of 100 µg/µl of anisomycin in ACSF or vehicle (ACSF) was injected using the autopump (Stoelting Co. Wood Dale, IL, 0.5 µl/min) and left in place for 2 minutes to allow for diffusion of the injectate. All animals received vehicle injections after surgery in Experiment 1 or after rehabilitation in Experiment 2 in order to assess the residual effects of isoflurane and the effect of injection procedures. In Exp1, two dosages (0.5 and 1.0 µl) of anisomycin were injected in order to assess the smallest amount necessary to disrupt reaching performance. No gross impairments in forelimb behaviors were found after anisomycin injections, such as failure to support body weight or perform reaching movements with the forelimb contralateral to the injection.

3.3.4 Intracortical microstimulation

All animals were anesthetized with a cocktail of ketamine (100 mg/kg) and xylazine (8 mg/kg). Supplemental ketamine (10mg per injection) and low levels of isoflurane (2-3%) were given when necessary. Bilateral craniectomies were performed over the motor cortex and small punctures in the cisterna magna were made before peeling the dura to prevent brain edema. Warm silicone oil (37 °C) was then put on top of the exposed cortex. One digital image (per hemisphere) of the exposed cortical surface was taken while a grid (500µm), used to serve as a guide, was superimposed over the image. A pulled glass pipette and platinum wire stimulating electrode (filled with 3M NaCl and

controlled by a hydraulic microdrive) was used to make systematic penetrations on the exposed cortex. The tip of the electrode was lowered to approximately 1500 μm. Stimulation with 1 Hz pulses from an isolated electric circule was used, and a non-response was recorded if no movement was observed with 50 μA of stimulation. The forelimbs were supported throughout the observations. Forelimb movements were classified as distal (wrist and digits) and proximal (elbow). Jaw, whisker, trunk, and hindlimb movments were all categorized as results of stimulation. The cortical area covered by a single penetration with electrical stimulation was 0.25 mm^2, and the forelimb representation map was determined from the data. Animals were overdosed with pentobarbital at the end of the procedures.

3.3.5 Single pellet skilled reach training and probe test

All the methods of reach training were the same as those previously described in chapter 2, including the initial shaping, pre-operative training, and post-operative rehabilitation periods. Pre-operative training with the preferrred limb was performed to ensure equivalency of reaching proficiency and rate of improvement between groups (which was used to match groups). Post-operative rehabilitation training and/or probe trial testing was with the pre-operatively preferred limb and, in lesion animals, the impaired forelimb. In Experiment 1, animals in both groups received 30 trials of the single pellet retrieval task on each one of the pre-operative training and post-infusion testing days (2~3 days following vehicle and anisomycin infusion). In Experiment 2, each pre-operative training session, probe test, and post-operative rehabilitative training session consisted of 30, 10, and 60 reaching trials respectively (same as chapter 2). The probe test sessions

consisted of only 10 reaching trials in order to prevent the lesion animals in the no rehabilitation group from experiencing rehabilitation effects for reaching performance. All animals received one probe test (probe 1) 4 days after surgeries and once a week for 3 weeks thereafter (probe 2-4). One day after probe 4, all animals then received vehicle injections followed by two 30-trial reaching tests (Post-VEH probes). The first of these two reaching tests was performed immediately following injections while the other was performed one day later. There was no significant difference in reaching performance between the two tests within subjects, and data were then combined for statistical analysis. One day after the second reaching test for vehicle injection, anisomycin and vehicle injections were made for the designated groups and all animals were tested 2 hours later with one post-injection test (Post-ANI probe: 30 trials).

3.3.6 Histological methods

Animals were anesthetized with a lethal dose of sodium pentobarbital and perfused intracardially with 0.1M phosphate buffer followed by fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer) one day and one hour after the last probe testing session in Experiment 1 and Experiment 2 respectively. Brains were extracted, placed in fixative solution and then sliced with a Leica VT1000S vibratome. Six rostral-to-caudal sets of 50 μm coronal sections were acquired throughout the cerebrum and then 5 sets were stored in cryoprotectant solution at -20°C until used. One set of sections for each brain was mounted onto a slide immediately after slicing and Nissl stained with toluidine blue for cortical volume measurement. A free-floating

immunocytochemistry method for synaptophysin was used for another set of sections in both experiments.

3.3.7 Remaining cortical volume estimates

The volumes of remaining cortex within the SMC region of the lesioned hemisphere and of the contralateral cortex were estimated to determine whether the tissue loss was similar in the three lesion groups. The sampling scheme focused on the region targeted by the lesion, starting with the appearance of the head of the caudate as the first of seven sections spaced 600 μm apart. For the infarcted cortex, the measurements included all remaining non-necrotic/non-gliotic cortical tissue. The volume within the SMC region was estimated using the Cavalieri method (Gundersen *et al.*, 1988). The area of remaining cortex in each section was measured using Neurolucida (MicroBringtField, Colchested, VT) perimeter tracing software. Volume was calculated as the product of the total area (summed over all sections) and the distance between section planes.

3.3.8 Immunocytochemistry

Immunocytochemistry studies for synaptophysin (see discussion 3.5.3) were done in both experiments, to assay whether PSI disrupted synaptic connectivities in either SMC in intact animals or the perilesion cortex in lesion animals to the extent that this can be assayed by this presynaptic vesicle protein. One set of sections was placed free-floating in 0.01 M phosphate-buffered saline solution (PBS) overnight at 4°C and transferred to 0.3% hydrogen peroxide in PBS for 30 min at room temperature to inactivate endogenous peroxidase activity. After several PBS washes, sections were incubated in a block

solution at room temperature for 2 hr to prevent non-specific protein binding. The block solution included 0.4% Triton X-100, 0.1% bovine serum albumin, and 2 % horse serum. After rinses in PBS, sections were incubated at 4℃ for 48 hr in mouse anti-synaptophysin (1:200, Sigma, St. Louis, MO) primary antibody in block solution. After 48 hr primary antibody incubation, sections were rinsed several times in PBS and then transferred to secondary antibody (1:200 biotinylated horse anti-mouse IgG) in 2 % horse serum in PBS for 1 hr at room temperature. After incubation, sections were again rinsed and incubated for 1 hr in a peroxidase-linked avidin-biotin complex (ABC kit, Vector Labs, Burlingame, CA). Immunoreactivity (IR) was then visualized using 3-3' diaminobenzidine tetrahydrochloride with nickel ammonium sulfate intensification procedures. The immunocytochemistry runs included sections incubated without the anti-synaptophysin primary antibody to verify the specificity of antibody labeling. The no-primary control sections did not contain evidence of distinct process staining. Sections were coded to conceal the experimental condition prior to quantification.

3.3.9 Quantification of synaptophysin labeling

Images from 5 coronal sections per brain were captured using a standardized light microscope and a high-resolution digital camera (x 84 final magnification). Layer II/III and layer V in the ipsilateral-to-injection hemispheres were outlined using cytoarchitectural landmarks and the luminosities were measured by the histogram feature of Adobe Photoshop. Optical densities (OD) were calculated as an inverse of the luminosity of tissue. Luminosities of contralateral homotopic areas were also measured as control structures. Statistical analyses verified that there were no group differences in

the OD of the control structures in either experiment. The data shown are the ratios between ipsilateral to contralateral-to-injection hemispheres in layer II/III or layer V in both the SMC and adjacent area (Exp. 1) or layer V in the perilesion cortex (Exp. 2).

3.3.10 Statistical analysis

SPSS (SPSS, Inc.) repeated-measures analyses of variance (ANOVAs) for the effects of Groups, Conditions, and Group by Condition interaction were used to analyze the behavioral measures. One-way ANOVAs were used for post hoc analyses when needed to further analyze group differences in behavioral performance on each day. ICMS forelimb representation map data were analyzed using repeated-measure ANOVA with Condition (pre- and post-injection) as a factor. There were no Order of injections or Hemisphere by Condition interaction effects between ANI-SMC and ANI-Adj groups ($F(1,6)=1.81$, $p=0.23$), so a one-way ANOVA was used for analyzing differences (SMC vs Adj) in the reduction in the forelimb representations caused by the anisomycin injection sites. Anatomical data were analyzed using one-way ANOVAs with Group as a factor.

3.4 RESULTS

3.4.1 Experiment 1: The effect of PSI in the forelimb SMC on forelimb motor representations and learned reaching performance in intact rats

3.4.1.1 Protein synthesis inhibition in the SMC but not adjacent area disrupts learned skilled reaching performance in intact rats.

Figure 3.2 shows the percentage of successful retrievals in the skilled reaching task. All rats were trained to a plateau and then received cannula implantation surgeries. Following surgeries, no impairments or disruptions of skilled reaching performance were found in either group in 2 testing sessions ("Post-OP" in Figure 3.2). Both groups then received vehicle (ACSF), 50 μg, and 100 μg anisomycin injections at separate time points. There were no significant Group by Time interaction effects between the first two testing sessions, 2 and 24 hrs after injections. The data for 2 and 24 hrs were then combined for repeated-measures ANOVAs that analyzed the effects of Groups (SMC vs Adj), Conditions (vehicle, 50 and 100 μg of anisomycin), and Group by Condition interactions. The results showed that there were significant Group by Condition interaction ($F(2,22)=12.79$, $p<0.001$) and Condition ($F(2,22)=10.23$, $p<0.001$) effects but no main effect of Group. Post-hoc analyses using one-way ANOVAs indicated that reaching performance was significantly different after the injection of vehicle and 100 μg anisomycin in the SMC group ($F(1,12)=10.70$, $p<0.01$). Furthermore, the reaching performance was only disrupted 2 and 24 hours (p's <0.05) after injections of 100 μg

anisomycin in the center of the SMC whereas there were no disruptions following adjacent injections. The disruption was no longer evident 48hrs after injections.

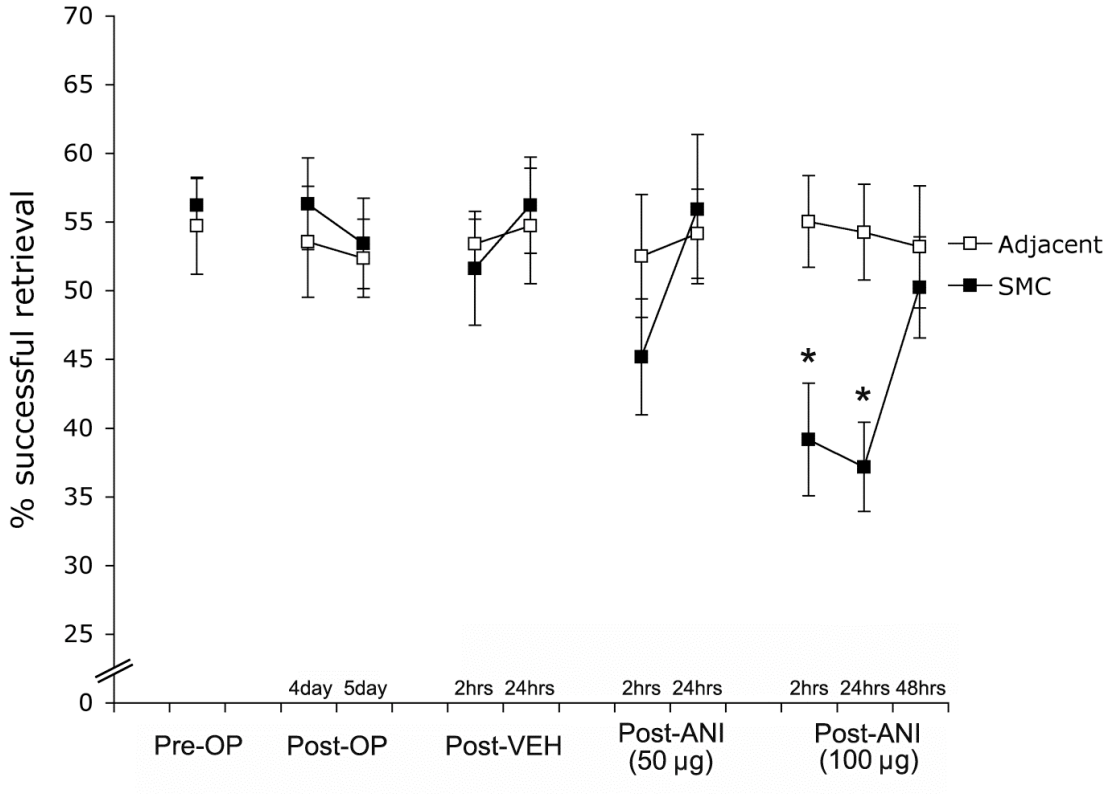

Figure 3.2 Skilled reaching performance in intact animals with injections of anisomycin in the center of the SMC or the adjacent area.

The percentage of successful retrievals in the skilled reaching task is shown. No disruption of skilled reaching performance was found after cannula-implantation surgeries (Post-OP). Both groups then received vehicle (ACSF), 50 µg, and 100 µg anisomycin injections at sequential time points followed by reaching tests. Reaching performance was disrupted 2 and 24 hours after injection of 100 µg anisomycin in the center of the SMC until 48hrs after injections. This was not found after anisomycin injections into the adjacent cortex (the " perilesion" cortex of Experiment 2). Data are means ± SEM. *p <0.05 significantly different from anisomycin injection in adjacent area.

3.4.1.2 Protein synthesis inhibition in the SMC and adjacent area cause different magnitudes of loss of forelimb movement representations.

Figure 3.3A shows the topography of movement representations in the SMC before and after injections in VEH, ANI-SMC, and ANI-Adj groups as revealed by intracortical microstimulation (ICMS). Either vehicle or anisomycin injections into cortical layer V of the SMC or anisomycin injections into cortical layer V of the area adjacent to the SMC were made immediately after the pre-injection mapping session. Thirty minutes follwing injections, post-injection mapping sessions were performed. Figure 3.3B shows the cortical area of forelimb movement representations before and after injections. Repeated-measures ANOVAs for analyses of each pair of maps with Condition (pre- and post-injection) as a factor revealed that the cortical area of forelimb representation was significantly decreased in both anisomycin injection groups (ANI-SMC: $F(1,3)=181.89$, $p<0.001$; ANI-Adj: $F(1,3)=13.05$, $p<0.05$). A one-way ANOVA revealed that the reduction of forelimb representations was significantly greater in the ANI-SMC group compared to the ANI-Adj group ($F(1,6)=35.00$, $p<0.01$). The injections of anisomycin into the center of the SMC resulted in a 96.88 ± 3.13% decrease in the cortical forelimb representation area that was measured prior to injection, while injections of anisomycin into the area adjacent to the SMC decreased by 55.00 ± 6.24%.

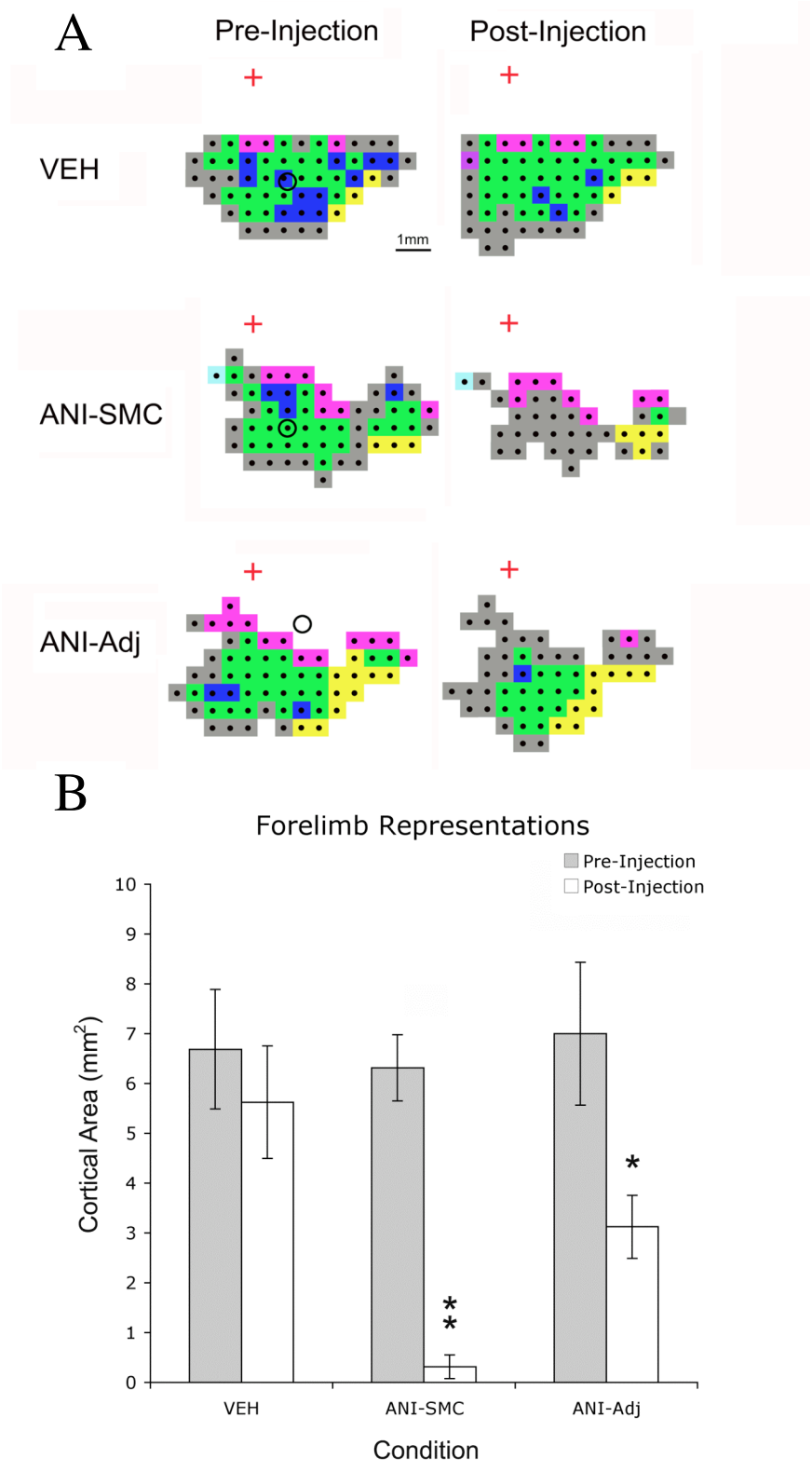
A
Pre-Injection
Post-Injection
VEH
1mm
ANI-SMC
ANI-Adj
B
Forelimb Representations
Pre-Injection
Post-Injection
Cortical Area (mm2)
10
9
8
7
6
5
4
3
2
1
0
*
**
VEH
ANI-SMC
ANI-Adj
Condition

Figure 3.3 Representative topography and cortical area of forelimb movement representations in the SMC.

(A) Representative motor cortical maps from animals that received vehicle or anisomycin injections in the center of the SMC or anisomycin inthe area adjacent to the SMC before and after injections. Distal representation (wrist and digits) are shown in green, proximal (elbow) in blue, vibrissae in pink, jaw in yellow, and no-response in gray. Black circles and red crosses represent injection sites and bregma, respectively. (B) The total area of forelimb representations before and after injections of vehicle (n=4), anisomycin in the SMC (n=4), and anisomycin in the adjacent area (n=4). Injections of anisomycin either in the center of the SMC or the area adjacent to the SMC resulted in decreases of the representation cortical area. SMC injections resulted in a greater loss than adjacent injections. Data are means ± SEM. $^{**}p<0.001$, $^{*}p<0.05$ significantly different from pre-anisomycin injection.

3.4.1.3 Protein synthesis inhibition did not alter synaptophysin labeling 72 hours after injection.

Table 3.1 shows the ratio of the optical density of synaptophysin labeling between the ipsilateral to contralateral-to-injection hemispheres. There were no significant differences in optical density between the two groups (SMC vs ADJ) 72 hours following injection in either layers II/III or V in the SMC or adjacent area (p's >0.05).

Table 3.1 Ratio of synaptophysin labeling

Sampling Area		SMC group	ADJ Group
Layer II/III	SMC	0.96 ± 0.04	1.08 ± 0.04
	Adjacent	0.97 ± 0.03	0.99 ± 0.04
Layer V	SMC	0.99 ± 0.03	1.03 ± 0.03
	Adjacent	1.01 ± 0.04	1.04 ± 0.02

Data are mean ± SEM ratios between ipsilateral to contralateral-to injection hemispheres

3.4.2 Experiment 2: The effect of PSI in the perilesion cortex on rehabilitation-induced functionality in rats with unilateral cortical infarcts

3.4.2.1 Lesion sizes were similar between groups

Figure 3.4 shows a representative sequence of a SMC lesion and cannula tract, and a gross view of the lesion brain. Measurement of the volume of the remaining SMC and contralateral hemisphere indicated that lesion sizes were similar in the three groups. The remaining volume of lesion cortex in mm^3 (mean ± SEM) was 84.10 ± 1.74 in the ANI group, 81.96 ± 2.69 in the VEH group, and 84.78 ± 3.03 in the Par group. The volume of contralateral SMC was 91.77 ± 1.63 in the ANI group, 89.70 ± 1.60 in the VEH group, and 90.49 ± 2.23 in the Par group.

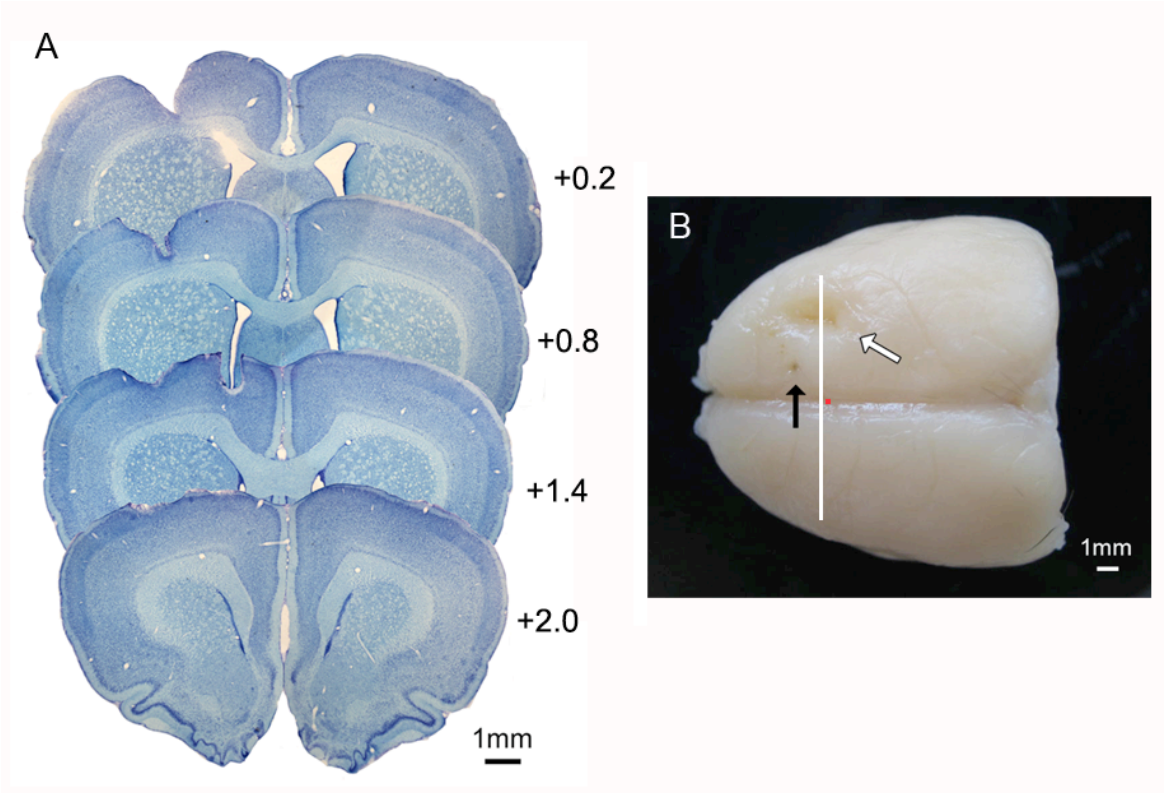

Figure 3.4 Representative sequence of a SMC lesion and cannula tract and gross view of a lesioned brain.

(A) Representative set of Nissl stained coronal sections. The cannula-implantation tract was confined to a limited region. Numbers to the right are approximate coordinates (mm) relative to bregma. (B) Gross view of lesioned cerebrum. The black arrow indicates the cannula tract and the white arrow indicates the SMC lesion. Bregma is indicated by a red dot. The white line indicates the plane of a section 0.2mm anterior to the bregma, which corresponds to the topmost coronal section in (A).

3.4.2.2 Protein synthesis inhibition in the perilesion cortex inhibits the recovered skilled reaching performance of rats that received rehabilitation following unilateral ischemic lesions.

Figure 3.5A shows the percentage of successful retrievals in skilled reaching performance. All rats were trained to a plateau and then received unilateral ischemic lesions and cannula implantation surgeries. Following surgeries, a decline in skilled reaching performance was found in all groups (probe 1). Then all groups, except the small NoRT group, began to receive rehabilitation for 3 weeks. Probes 2, 3, and 4 were done after the 1^{st} , 2^{nd}, and 3^{rd} weeks of rehabilitation respectively. By using repeated-measures ANOVAs with Groups and Time as factors, there were no significant differences in daily reaching performance (data not shown) and weekly probe tests (probe 1 to 4) between the three groups that received rehabilitative training over the 3 week span. Time effect ($F(3, 51)=21.85$, $p<0.001$) was significant, however, reflecting that skilled reaching performances improved during rehabilitation. The reaching performance data of probe tests for the three groups receiving rehabilitation were combined (n=20) and then compared with the no rehabilitation training pilot animals (NoRT, n=4). The analyses showed that there were significant Group by Time interaction effects ($F(3,66)=5.14$, $p<0.01$), consistent with results from chapter 2. Post-hoc analyses using

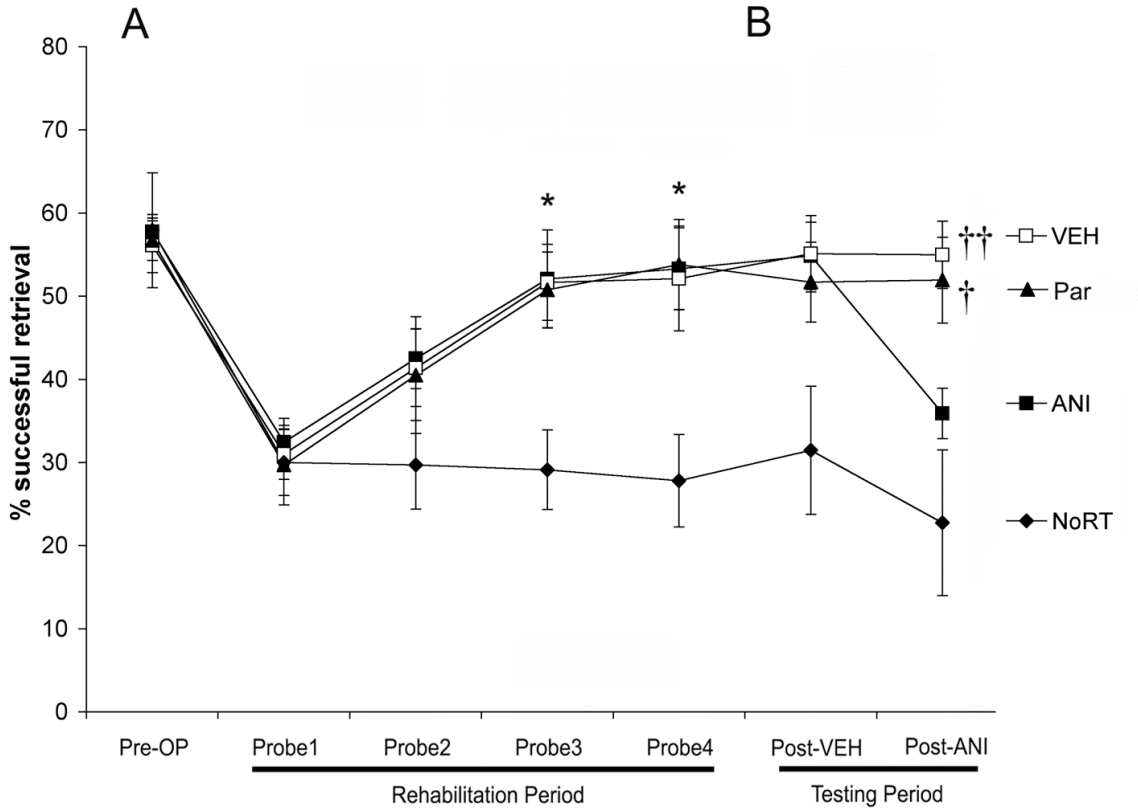

Figure 3.5 Skilled reaching performance in animals with unilateral ischemic lesions following rehabilitation training was disrupted by injections of anisomycin in the perilesion cortex.

The percentage of successful retrievals in skilled reaching performance is shown. (A) Impairment of skilled reaching performance was found after surgeries. The three groups that received rehabilitation had better reaching performances on probes 3 and 4 compared to a small set of no rehabilitation controls, replicating previous findings (Chapter 2). (B) A significant difference in the disruption of skilled reaching performance was found in the ANI group after anisomycin injections in the perilesion cortex in comparison with the VEH group after ACSF injections and the Par group after anisomycin injections. Data are means ± SEM. *$p<0.01$ significantly different from no rehabilitation controls. †$p<0.05$, ††$p<0.01$ significantly different from ANI group.

one-way ANOVAs indicated that the animals receiving rehabilitation had better reaching performances in Probes 3 and 4 compared to no rehabilitation controls (p's<0.05).

Repeated-measures ANOVAs with Group and Condition (Post-VEH and Post-ANI probes) as factors revealed significant Condition ($F(1,17)=19.82$, $p<0.001$) and Group by Condition ($F(2,17)=22.23$, $p<0.001$) effects between the three rehabilitative groups (as shown in Figure 3.5B). Post-hoc analyses using one-way ANOVAs indicated that the disruption of skilled reaching performance in the ANI group after anisomycin injections was significantly different from the disruption seen in the VEH group after ACSF injections ($F(1,13)=14.84$, $p<0.01$) and the Par group after anisomycin injections ($F(1,13)=8.07$, $p<0.05$).

3.4.2.3 Protein synthesis inhibtion in the perilesion cortex did not alter synaptophysin labeling two hours after injection.

The ratios of the optical densities of synaptophysin labeling in layer V of the perilesion cortex (near the tip of cannula implantation tract) and of the contralesional hemisphere were analyzed. There were no Group effects between the three rehabilitative training groups (ANI, VEH, and Par) at two hours after injections ($p>0.05$). The ratio between the two hemispheres (mean $\pm$ SEM) was 1.17 ± 0.06 in the ANI group, 1.25 ± 0.14 in the VEH group, and 1.03 ± 0.02 in the Par group.

3.5 DISCUSSION

The maintenance of skilled reaching performance in intact animals has been shown to depend on continuous protein synthesis (Kleim *et al.*, 2003a). In the present study, PSI induced by an injection of anisomycin in the forelimb SMC both impaired learned reaching performance and resulted in a loss of the forelimb motor representation region. While in intact rats PSI in the area adjacent to the SMC did not disrupt learned reaching performance, PSI in the corresponding area in rats with unilateral ischemic lesions did impair regained reaching performance.

3.5.1 PSI in the forelimb SMC disrupts the forelimb motor representation map and learned reaching performance in intact rats.

PSI induced by anisomycin injection in the SMC disrupted learned skilled reaching performance and caused a loss of forelimb representations, as revealed by intracortical microstimulation, in intact rats. These findings replicate those of a previous study by Kleim and colleagues (2003a) that indicated that continuous protein synthesis is required to maintain labile functional organization in the SMC and that the disappearance of the motor map is linked with impairments in skilled forelimb movement. In order to rule out the possibility that the inhibitory effects of anisomycin were more global in nature, injections of anisomycin were also made in the area adjacent to the SMC, where structural reorganization takes place following an ipsilateral SMC ischemic lesion. Even though PSI in the area adjacent to the SMC disrupted approximately half of the forelimb representation map that was closer to the injection site, it did not interfere with

learned skilled reaching performance. These results suggest that a focal injection of anisomycin disrupts a confined area of forelimb representations in the SMC, and that learned skilled reaching performance is not disrupted with only a partial (approximately half) loss of the forelimb representation map. Thus, the focal injection of anisomycin could be used as a tool to test the role of the reorganized perilesion cortex in the regaining of function after cortical ischemia.

Several studies have investigated the varying degrees of PSI induced by injections of anisomycin at different time points. Rosenblum *et al.* (1993) found that the effect of PSI peaks approximately 30 minutes after injection and slowly decreases thereafter. Kleim *et al.* (2003a) showed 50% PSI 20 min after injection, and Luft *et al.* (2004) found 84% PSI one hour after injection and the inhibition fully abated after 48 hours. It was found that the disruption of forelimb representations coincided with synapse loss at 4 days after anisomycin injection, and that skilled reaching performance was also disrupted at that time point in rats that had not received any skilled testing since injection (Kleim *et al.*, 2003a). The present results showed that the disruption of skilled reaching performance caused by PSI in the SMC had disappeared at 48 hours after anisomycin injection. In the present study, all animals were tested at three separate times,receiving a total of 90 trials in the skilled reaching task within 48 hours of injection. PSI had been shown to block motor learning in a novel task for 4-6 days (Kleim *et al.*, 2003a; Luft *et al.*, 2004). It had also been found that the anisomycin-induced loss of motor map and disruption of reaching performance are reversed with skilled motor training (Kleim *et al.*, 2003a), a task that had been shown to increase the synaptogenesis that is co-localized with expanded forelimb motor maps (Kleim *et al.*,

2002). The present results extend these findings by suggesting that recovery from a disruption in the performance of an already learned task occurs sooner than recovery from a disruption in learning a new task by skilled motor training.

3.5.2 PSI in the perilesion cortex disrupts the functional recovery induced by rehabilitative training after unilateral cortical infarct in rats.

Consistent with previous studies (*e.g.* Biernaskie an Corbett, 2001; Conner *et al.*, 2005) and the findings in Chapter 2, all animals with unilateral ischemic SMC lesions in this study improved in reaching success after motor rehabilitative training compared to pilot control animals that received no rehabilitation. After recovered skilled reaching performances reached a plateau, PSI in the perilesion cortex negated those recovered skilled reaching performances, while PSI in the distal parietal cortex, which was designed as a control intervention, did not show similar disruptions. These findings demonstrate that the effects of PSI on functional recovery in the skilled reaching task are specific to the perilesion cortex. As discussed in the last section, PSI in the area adjacent to the SMC in intact animals disrupted a portion of the animals' forelimb representation maps, yet the animals maintained their ability to perform a learned skilled reaching task. It has also been found that motor maps expand into the remaining tissue of the perilesion cortex after a focal cortical injury (Conner *et al.*, 2005). These findings suggest that the reorganized perilesion cortex is functionally critical to the recovery of skilled reaching performance.

3.5.3 PSI induced disruption of skilled reaching performance did not correspond with labeling of the presynaptic vesicle protein, synaptophysin.

Synaptophysin is an integral membrane glycoprotein in pre-synaptic vesicles that is present in nearly all neurons in the brain that participate in synaptic transmission (Thiel, 1993). Synaptophysin labeling is now commonly used for quantification of the presynaptic component of synaptic plasticity measurements. It has been found that synaptophysin increases after exposure to an enriched environment in mice (Nithianantharajah et al, 2004) and following motor skill learning in rats (Derksen *et al.*, 2007). Furthermore, it is involved in the modulation of the synaptic plasticity that is associated with whisker stimulation in rats (Ishibashi, 2002).

In Experiment 1, I failed to find a difference between the two testing groups (SMC vs ADJ) in the labeling of synaptophysin in layers II/III or V of the SMC at seventy two hours after anisomycin injection in the SMC (SMC group) or the area adjacent to the SMC (ADJ group) in intact rats. In Experiment 2, it was found that anisomycin injections in the perilesion cortex did not alter synaptophysin labeling. The finding in Experiment 1 is consistent with the behavioral outcome that anisomycin transiently disrupted skilled reaching performance. It is possible that the transience of the effects of anisomycin on skilled reaching performance and the lack of difference in synaptophysin levels at 72 hours post-injection was due to a relearning of the skilled reaching task during the 90 tests performed after injections. It is also possible that PSI effects on structural synaptic proteins mainly occurred in the post-synaptic component, which would not be detected by synaptophysin labeling. For example, PSI might decrease the levels of postsynaptic density (PSD) proteins and result in synaptic

weakening. Ehlers (2003) indicated that a specific group of PSD proteins are rapidly turned over via the ubiquitin-proteasome system and that this turnover is sensitive to synaptic activity. These findings could help explain why motor maps had disappeared after 30 min and the learned or regained reaching performances were disrupted within 2 hours. Other effects of anisomycin, such as the activation of mitogen-activated protein kinases (MAPK) p38 (Hazzaline *et al.*, 1998) and c-Jun N-terminal protein kinease (JNK), which regulates the release of proapoptotic mitochondrial factors (Chauhan *et al.*, 2003), are also likely to contribute to neuroplasticity.

By sampling every ten minutes, it has been found that synapses appear and disappear rapidly in the developing barrel cortex (Lendvai *et al.*, 2000). In the adult barrel cortex, it has been found that some synapses are constantly being turned over; however, only a small proportion of synapses can be rapidly turned over, while 80% of synapses last for a day or longer and 60% last for at least 8 days (Trachtenberg *et al.*, 2002). Kleim *et al.* (2003a) found that the size and number of synapses in the SMC were reduced four days after anisomycin injection, and that this coincided both with a loss of motor map, as revealed by ICMS, and with the disruption of skilled reaching performance. Thus, it is still possible that PSI results in a net decrease in the total synapse number over a period of time by preventing new synapse formation.

Failure to find a decrease in synaptophysin labeling might due to other reasons. Some possibilities include that synaptophysin is not sensitive to anisomycin in the adult motor cortex, that synaptophsyin levels might be compensated for by protein synthesis remote from the injection site (*e.g.* the cell soma), or that the current optical density measure is not sensitive enough to reveal the significance. Further investigation is

necessary to determine which of the proteins (*e.g.* PSD proteins, JNK, MAPK p38) that are decreased by PSI might be responsible for the loss of synapses reported by Kleim *et al.* (2003a).

3.5.4 Implication for the role of the perilesion cortex in functional recovery induced by rehabilitation

Reorganization of motor representations in the perilesion cortex has been found following a focal SMC lesion and is linked with functional recovery (Castro-Alamancos and Borrel, 1995; Conner *et al.*, 2005). As mentioned in Chapter 2, the synaptic plasticity found in the perilesion cortex might mediate the reinstatement of the neural integrity and reorganization of circuitry that underlies the functional improvements. The present results extend these previous findings by showing that transient protein synthesis inhibition in the perilesion cortex reinstates functional deficits. This strongly supports the importance of the perilesion cortex in recovery of skilled reaching performance. While the present findings support the necessity of this area, they of course do not indicate sufficiency. It seems very possible that the rehabilitation-induced plasticity found in the perilesion cortex represents just one aspect of plasticity that occurs across a network of the somatosensory and motor systems. Further research is needed to identify the specific proteins that are involved in the cellular mechanisms that mediate structural and functional changes within the reorganized cortex as well as how this reorganization may be coordinated with plasticity of motor cortical connections.

Chapter 4: Motor cortical stimulation with rehabilitation enhances peri-infarct synaptic plasticity following sensorimotor cortex lesions

4.1 ABSTRACT

Cortical stimulation (CS) as a means to modulate regional activity and excitability in cortex is emerging as a promising approach for facilitating rehabilitative interventions after brain damage, including stroke. The aim of this study is to determine whether a treatment that enhances the efficacy of rehabilitative training also enhances perilesion synaptogenesis. We investigated whether synaptic plasticity in peri-infarct cortex is linked with CS-induced functional improvements. This analysis used a set of animals from a previous study of CS effects (Adkins, 2005) which provided further support that CS combined with rehabilitation improves skilled reaching performance in rats. In these rats, stereological electron microscopy methods were used to quantify axodendritic synapse subtypes in layer V of the motor cortex underlying the electrode, which was the region found to undergo rehabilitation-induced synaptic plasticity in Chapter 2. The results indicate that both severely and moderately impaired CS subgroups had significantly greater densities of axodendritic synapses in the stimulated cortex compared to impairment-matched NoCS groups (increased by 12% and 22% respectively), and moderately impaired CS rats had increases in presumed efficacious synapse subtypes (perforated synapses: 86%; multiple synapses: 79%) compared to NoCS. Synaptic density was positively correlated with post-rehabilitation reaching success. These

findings suggest that CS-induced functional improvements may be mediated by synaptic structural plasticity in stimulated cortex.

4.2 INTRODUCTION

Motor impairments are among the most common disabilities caused by stroke (Thom *et al.*, 2006). Motor rehabilitative training can reduce these impairments but it is often insufficient to restore normal levels of function (Duncan *et al.*, 2000; Dobkin, 2004). Recent studies in humans indicate that stimulation of select cortical regions might be used to improve the efficacy of rehabilitative training (Brown and Pilitsis, 2006; Hummel and Cohen, 2006; Pascual-Leone, 2006). In support of this possibility, recent studies in monkeys (Plautz *et al.*, 2003) and rats (Adkins-Muir and Jones, 2003; Kleim *et al.*, 2003b; Teskey *et al.*, 2003; Adkins *et al.*, 2006b) with sensorimotor cortex (SMC) infarcts indicate that the efficacy of rehabilitation can be greatly enhanced by coupling it with electrical stimulation (CS) of the peri-infarct cortex. In this approach, as lesion animals undergo daily training on a skilled reaching task, a subthreshold (to evoke movements) current is passed through electrodes positioned over the remaining motor cortex. Also, in a recent multicenter safety trial, CS combined with rehabilitative training was found to result in motor functional improvements of the stroke-affected hand in humans (Brown *et al.*, 2006). The neural mechanisms of these functional effects are unknown, but we hypothesize that CS recruits neurons that may otherwise be insufficiently activated during task performance and that this enables the activity-dependent synaptic plasticity that mediates recovery of skilled movements in the impaired forelimb. Many studies had shown that CS-induced improvements in reaching

success coincide with neuroplastic changes in the stimulated region of the SMC, including increased surface density of layer V dendritic processes (Adkins-Muir and Jones, 2003), expansion of movement representations detected using intracortical microstimulation mapping (in rats: Kleim *et al.*, 2003b; in monkeys: Plautz *et al.*, 2003) and enlargement of the polysynaptic component of motor cortical evoked potentials (Teskey *et al.*, 2003) compared to animals receiving rehabilitation alone. Because plasticity of synaptic connectivity is thought to underlie motor learning and re-learning (Monfils *et al.*, 2005), a major purpose of the present study was to directly determine whether training combined with CS induces greater structural plasticity of synapses in remaining motor cortex than training alone. Furthermore, because the severity of motor impairment following stroke in humans is a major variable in the effectiveness of rehabilitation (Duncan *et al.*, 2000), another goal of the present study was to determine whether CS effects on structural plasticity of synapses vary with the severity of the injury-induced impairment in forelimb function.

After pre-operative training to criterion on a skilled reaching task rats received SMC lesions and implantation of electrodes over the remaining motor cortex. Beginning two weeks post-infarct, animals received eighteen days of training and testing on the skilled reaching task with (CS) or without (NoCS) stimulation. As shown in Figure 4.1, Adkins (2005) found that moderately, but not severely impaired rats, had better recovery of reaching success following rehabilitation with CS. Stereological methods and transmission electron microscopy were then used to quantify synaptic density in layer V of the motor cortex underlying the electrode, including synapse subtypes that have been

implicated in the enhanced synaptic efficacy that is thought to underlie learning (*e.g.*, Jones, 1999; Toni *et al.*, 2001; Ganeshina *et al.*, 2004; Connor *et al.*, 2006).

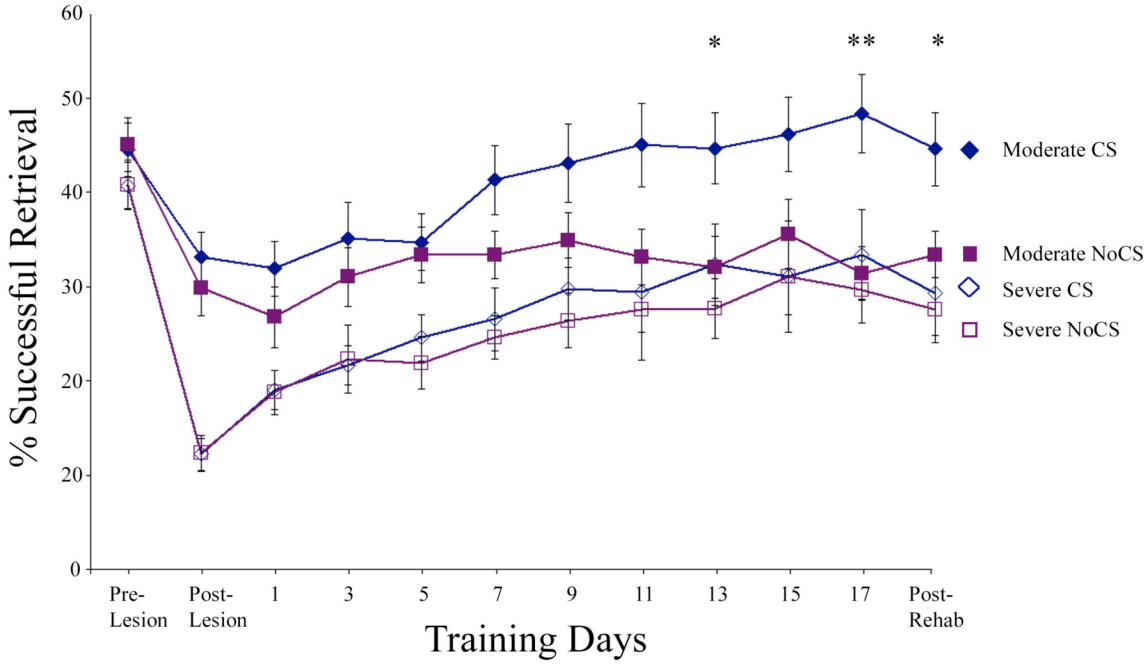

Figure 4.1 Cortical stimulation during rehabilitative training enhances reaching performance in moderately impaired animals.

All animals had impairments in reaching performance following SMC lesions and an increase in success level over the 18 days of practice in the single pellet reaching task. In groups with initial success rates of $\geq$ 20% after the lesions (Moderate), 100Hz epidural cortical stimulation (CS) given during rehabilitative training days significantly enhanced reaching success over days of testing compared to all other groups. CS did not produce performance enhancements in the animals with more severe initial deficits (< 20% success rates; Severe), although it did partially normalize the movements used for reaching in this subgroup (Data not shown). Data are means ± S.E.M. *$p < 0.05$,**$p < 0.01$ significantly different from Moderate NoCS. (From Adkins, 2005; Usage with permission of Dr. Adkins)

4.3 MATERIALS AND METHODS

4.3.1 Subjects and experimental designs

Forty-eight adult 3 to 4 months old male Long-Evans hooded rats were used for the behavioral study published before (Adkins, 2005). Grouping procedures are briefly mentioned here. Following post-lesion testing, animals were randomly divided into CS and NoCS groups with the exception that they were matched as closely as possible for pre- and post-operative performance. Animals then were further subdivided based upon their initial post-lesion (pre-rehabilitation) reaching performance so that there were four groups: severely impaired + CS (Severe CS), severely impaired + NoCS (Severe NoCS), moderately impaired + CS (Moderate CS), and moderately impaired + NoCS (Moderate NoCS). NoCS control groups received cortical implants as well, and animals were attached to stimulator cables during reach training without receiving any current. Stereological methods and transmission electron microscopy were performed in subsets of randomly chosen brains (Severe CS: n = 5, Severe NoCS: n = 7, Moderate CS: n = 9, Moderate NoCS: n = 9) to provide a sample adequate to detect significant differences resulting from CS.

4.3.2 Reach training methods

The single pellet retrieval task described in previous chapters was used in this study as rehabilitative training and assessment of reaching performance. Rats were trained for 60 trials per day or 15 min, whichever came first. Beginning fourteen days post-infarct,

animals were trained for 18 consecutive days, while receiving cathodal 100 Hz CS or no stimulation. Previous studies have found that the effects of CS are frequency dependent (Adkins-Muir et al., 2003; Teskey et al., 2003) and 100 Hz CS was chosen because this has been found to be particularly effective in improving reaching performance (Teskey et al., 2003). The intensity and duration of the reach training combined with CS were chosen because this combination was previously found to improve reaching function after SMC lesions (Adkins et al., 2006b). All rats were attached to stimulator cables and placed into the reaching chamber. For the CS groups, stimulation was delivered at 40-50% of movement threshold. Stimulation was initiated when animals first approached the reaching window and continued for the duration of the training session. No stimulation was delivered to the rats in the NoCS group. Reaching performance was measured as the percentage of successful retrievals out of the total number of reach attempts.

4.3.3 Histological measures

4.3.3.1 Tissue Processing

All data were collected from tissue that was coded to conceal the experimental condition. Within two days after the last training session, the thirty animals used in the electron microscope portion of the study were given an overdose of sodium pentobarbital and transcardially perfused with 0.1M sodium phosphate buffer followed by fixative in the same buffer (2% paraformaldehyde and 2.5 % glutaraldehyde). Alternating sets of 200, 100 and two 50 μm-thick sections of the cerebrum were cut using a vibratome and collected into fixative. The 50 μm sections were stained with Toluidine blue (Nissl) for lesion reconstruction and volume estimates in Adkins' study (2005). For electron microscopic analysis of synaptic density and light microscopic measures of neuronal density, non-necrotic/non-gliotic tissue in the perilesion motor cortical region underlying the electrode was sampled inclusive of the medial and lateral agranular region between 1.2 and 1.6 mm anterior to bregma (Figure 4.2). Using a stereomicroscope, this region was identified, using macrostructural landmarks and unique cytoarchitectural characteristics which are evident in unstained tissue (Jones *et al.*, 1999), and was removed in the 200 μm sections. All samples were then placed in cacodylate-buffered osmium tetroxide, and *en bloc* stained with 2% uranyl acetate for 45 min. Samples were then dehydrated and sandwich-embedded in Eponate-12 resin. Semithin sections (0.8 μm thickness) were then extracted, stained with Toluidine Blue and used to estimate neuronal density and to more precisely localize the region for electron microscopic

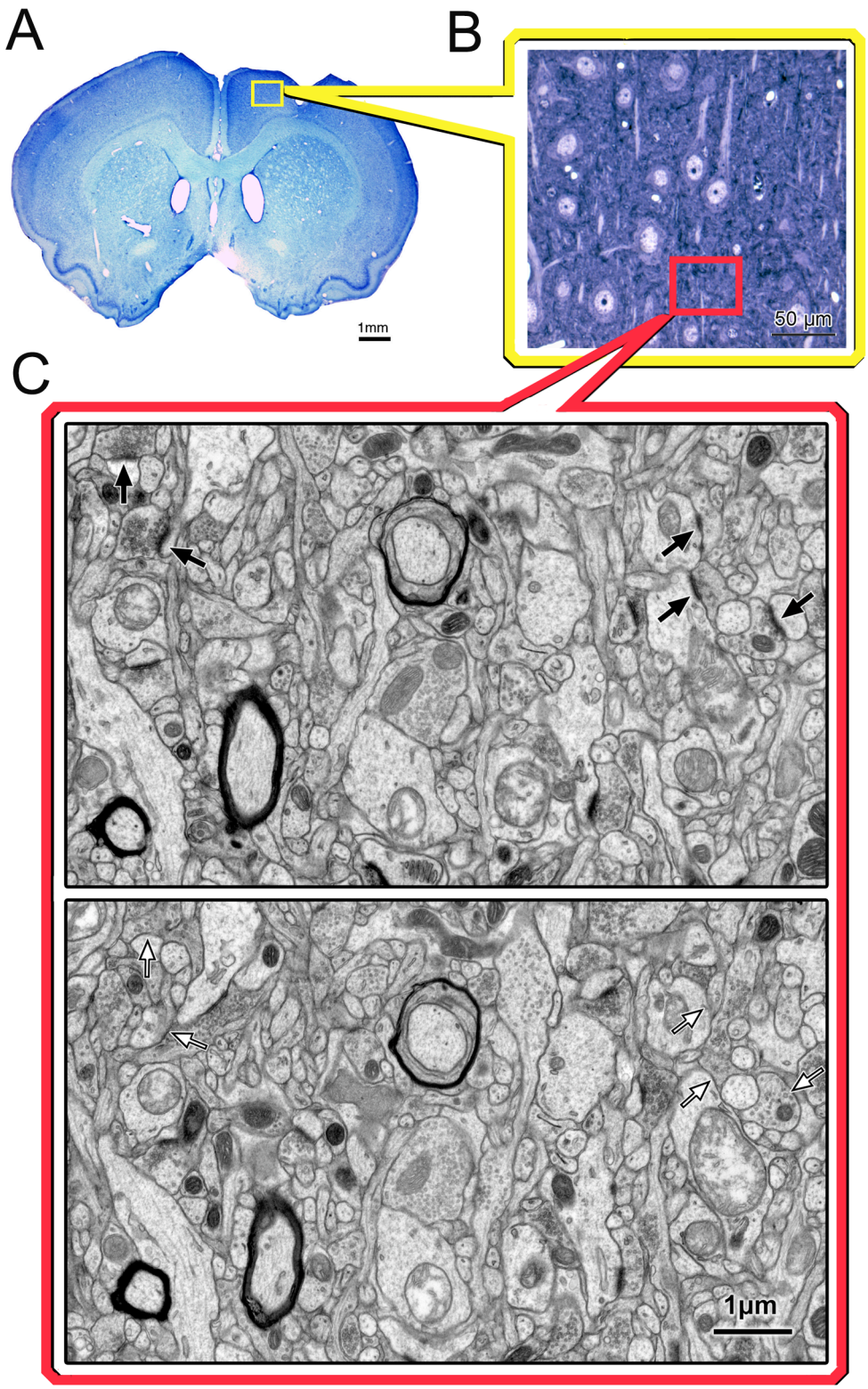
A
B
C
1mm
50 µm
1µm

Figure 4.2 Sampling strategy in perilesion motor cortex.

Changes in synaptic density in layer V of the perilesion cortex were assessed using the physical disector method for quantitative transmission electron microscopy. (A) The region sampled was the remaining agranular cortex near the rostral portion of the lesion. (B) Semithin sections were used for neuronal density estimates and to identify the sampling region for electron microscopy. (C) Disector pair of electron micrographs of layer V motor cortex. Arrows indicate postsynaptic densities that disappear from one section to the next and are therefore counted.

sampling. Serial silver gray ultathin (70 nm) sections were obtained from the osmicated samples using a Leica Ultracut R microtome, mounted onto slotted copper grids coated with formvar film and stained with lead citrate to be used for electron microscopic measures of layer V synaptic density. All histological data were collected by an individual blind to the experimental condition.

4.3.3.2 Neuronal Density Measures

The density of neurons in layer V of the perilesion cortex was estimated using the physical disector method (Gundersen *et al.*, 1988). Disector pairs used for neuronal density measures consisted of digital images taken from every other serial semithin section using a Nikon Optiphot-2 light microscope equipped with a rotating stage. The sampling strategy was intended to optimize the consistency of the sampling relative to both the lesion boundary and cortical subregions. Cytoarchitectonics in adjacent 50 µm thick sections and low magnification semithin images were used to localize the sample area to two subregions within layer V of the agranular cortex medial to the lesion and approximately 1.2-1.6 mm anterior to bregma. One subregion was near the medial

boundary of the lesion, excluding fibrotic and necrotic tissue. The second subregion was at least 600 μm medial from the first subregion and was located in the medial agranular cortex near the border of the cingulate cortex. Seven animals had more medial lesions (n=1 in Severe CS and n=2 in each of the other 3 groups) and thus data could only be collected from one subregion, which was both near the lesion boundary and in medial agranular cortex. The neuronal density in these seven animals was not significantly different from the rest of the animals ($F(1,28) = 0.74$, $p=0.40$). Images of 44,000 μm^2 layer V samples were taken using a high-resolution digital camera (DVC Co., Austin, TX) at a final magnification of 830X. Once the images from section 1 were captured, the same sample fields were found and captured in each of the next four sections of the series. The same sampling strategy was applied to another set of 5 serial sections, so that a total of 20 images (10 images in seven animals) were captured for each animal. Images from each adjacent set of sections were used as a disector pair and the neurons were counted if they were present in the "reference" section but not present in the "look-up" section (Gundersen *et al.*, 1988). All samples of each set of 5 serial sections were used as both a reference and look-up section so that 32 disector pairs were used per animal (16 pairs in animals with only medial samples). Unbiased sample frames were placed onto each image in Adobe Photoshop and neurons were identified by multiple criteria including the presence of a nucleus surrounded by cytoplasm and, frequently, the presence of a pyramidal shaped soma. The coefficient of errors (CEs; West and Gundersen, 1990) of the neuronal density estimates per rat ranged from 0.03 to 0.12 (median = 0.05) and mean CEs were similar between groups: 0.05 (Severe CS) to 0.06 (Moderate NoCS). Neuronal density was calculated by the formula: $Nv = \Sigma Q^- / \Sigma v_{(frame)}$, where: ΣQ^- is the sum of neurons counted per brain and $\Sigma v_{(frame)}$ is the sum of the sample volume (2,252,800 μm^3). The sum of the sample volume was calculated as

the product of the area of one sample frame, the distance between section planes (1.6 μm) and the number of samples (32). Layer V cortical volume per neuron was calculated as the inverse of neuronal density.

4.3.3.3 Synaptic measures

The densities of synapses in layer V, medial and rostral to the lesion, in the residual motor cortex were also estimated using the physical disector method. Four sets of four serial adjacent electron micrographic samples (final magnification of 14,000 X) spanning at least 11 sections were imaged from serial silver gray (70nm) sections. The first set of four serial images was taken in the lateral extent of the region sampled for neuronal density estimates. Moving medially, the first section of the next series was taken in the same section as the last section from series 1 and then from 3 additional sections. This was repeated for the next 2 series. This strategy minimizes the contribution of section-to-section variability in thickness. Occasionally, the presence of artifacts (folds, lead precipitant) required adjustment in the overlap of the series. The first sample of each series was positioned randomly with the exception that cell bodies, capillaries and large dendritic shafts (*e.g.*, the proximal apical shaft) were dodged. At each sample site, four 38 μm^2 digital images were taken with a Hamamatsu 1394 digital camera installed in a Philips 208 transmission electron microscope and then photomerged into a single digital electron micrograph in Adobe Photoshop. The axodendritic synapses were identified by the presence of a post-synaptic density and at least three vesicles in the presynaptic bouton (Figure 4.2). Each micrograph was used as both a reference and a look-up section for counting the post-synaptic densities so that there were 24 disector pairs per brain. The CEs of the synaptic density estimates per rat ranged from 0.026 to 0.056

(median = 0.038) and group means range from 0.037 (Severe CS) to 0.042 (Severe NoCS). Synaptic density was calculated by the formula: $Nv = \Sigma Q^{-} / \Sigma v_{(frame)}$, where ΣQ^{-} is the sum of synapses counted per brain and $\Sigma v_{(frame)}$ is the sum of the sample volume (188.05 μm^3). $\Sigma v_{(frame)}$ was calculated as the product of the area of one sample frame (112 μm^2), distance between section planes (70nm) and the number of samples (24).

4.3.3.4 Efficacious synaptic subtype measurement

Perforated (Perf) synapses and synapses formed by multisynaptic boutons (MSBs) were also estimated because these synapse subtypes have been linked with increases in synaptic efficacy (*e.g.*, Geinisman *et al.*, 1991; 2001) and have previously been found to increase in the motor cortex in association with the acquisition of motor skills (Kleim *et al.*, 1998; Jones *et al.*, 1999). Boutons forming synaptic contacts with more than one distinct dendritic element (spine or shaft) were identified as MSBs (se Figure 2.5). Synapses containing a distinct perforation or segmentation in the post-synaptic density (PSD) were identified as Perf synapses. MSB and Perf synapses were counted using the physical disector method as described above. Multiple short section series (4 sections/series) were used in this study to provide a greater breadth of sampling; however, this limits the ability to reconstruct boutons in three dimensions (which requires much longer section series) and greatly underestimates MSB and Perf synapse density (Jones *et al.*, 1997; Jones, 1999). It has previously been found that the measurement of MSBs and perforated synapses in short series of sections is as sensitive to group

differences in the prevalence of these synapse subtypes as reconstruction methods (Jones *et al.*, 1997; Jones, 1999).

4.3.3.5 Analysis of remaining agranular cortex within SMC region

Lesion extent and size were assessed previously by Adkins (2005, shown in Figure 4.3). For cortical volume measures, including all cortical tissue within coronal sections of the SMC region, ANOVA indicated no significant differences between brains processed for electron microscopic studies (n=30) and the others (n=18, $F(1,44) = 1.73$, $p<0.05$) and these data, when analyzed together, showed all lesions produced damage to the cytoarchitectually identified forelimb area (Wise and Donoghue, 1986) of the SMC. There were no major observable differences in extent or placement between groups. In order to aid in the interpretation of synaptic and neuronal density measures, the volume of layer V in the remaining agranular cortex within this SMC region was further measured using 3 consecutive Nissl stained sections (400μm apart) between ~0.8 and 2.0 mm anterior to bregma.

4.3.4 Statistical analysis

Anatomical data were analyzed using one-way ANOVA's to compare groups. Fisher's LSD post hoc analyses were used when needed to further analyze group differences. Bivariate correlations were used to assess the relationship between the synaptic density and reaching performance in the last training sessions. Significant levels were set at 0.05.

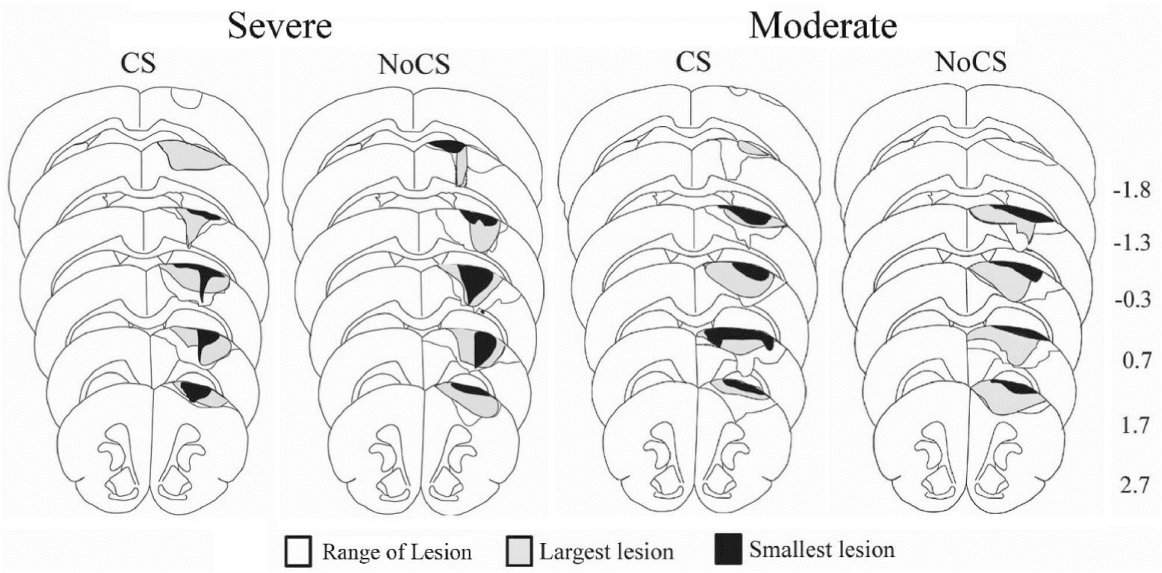

Figure 4.3 Reconstruction of the extent and placement of focal SMC lesions.

Reconstructions of each lesion are overlaid onto the left hemisphere of schematic coronal sections. There were no major differences in extent or placement between groups nor differences in remaining cortical volume. For each group, black outline indicates the range of all lesions, grey shading represents the largest lesions, and black shading represents the smallest lesion. Numbers to the right indicate approximate coordinates in mm rlative to Bregma. (From Adkins, 2005; Usage with permission of Dr. Adkins)

4.4 RESULTS

4.4.1 Motor cortical stimulation during rehabilitation increases synaptic density in layer V of perilesion cortex in rats.

Figure 4.4 shows the axodendritic synaptic density measured in the motor cortex medial to the lesion underlying the electrode. Synaptic density was significantly different between groups ($F(3,26)=8.92$, $p<0.001$). Post-hoc analysis indicated significantly increased synaptic density in CS treated rats compared with impairment-matched NoCS controls in both severely ($p=0.046$) and moderately ($p<0.001$) impaired animals. There were no significant differences between moderately and severely impaired subgroups. There were also no group differences in remaining cortical volume within the SMC region in this subgroup of rats (Adkins, 2005) or within layer V of the remaining agranular cortex ($F(3,26)=0.51$, $p=0.68$; mean ± SEM in mm^3 = 1.02 ± 0.21 in Severe CS, 0.87 ± 0.10 in Severe NoCS, 1.07 ± 0.13 in Moderate CS and 1.11 ± 0.15 in Moderate NoCS). Therefore, these increases in synapses most likely reflect net increases in synapse numbers. In the measure of neuropil volume per neuron, the main effect of group approached significance ($F(3, 26)=2.84$, $p=0.057$), reflecting a tendency for there to be greater layer V volume per neuron in the Moderate CS group (26799.08 ± 1217.09μm^3) compared to the other groups (*e.g.*, versus 23768.53± 776.00 μm^3 in Moderate NoCS). However, there were no significant differences in remaining neuronal number (neuronal density X layer V agranular cortical volume) between groups ($F(3, 26)=0.64$, $p=0.59$).

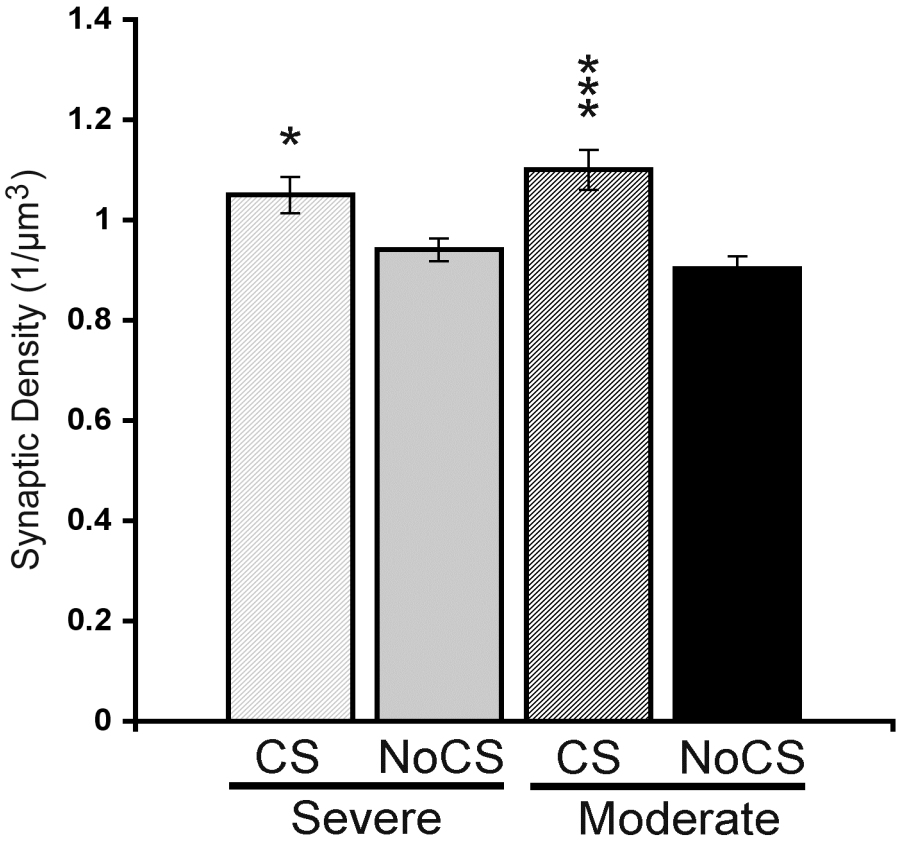

Figure 4.4 Axodendritic synaptic density was significantly different between groups.

Post-hoc analysis indicated significantly increased synaptic density in CS treated rats compared with impairment-matched NoCS controls in both severely and moderately impaired animals. Data are means ±S.E.M. *$p<0.05$, ***$p<0.0001$.

4.4.2 Motor cortical stimulation during rehabilitation increases efficacious synapse subtypes in perilesion cortex of moderately impaired rats.

Figure 4.5 shows the density of synapses with perforated post-synaptic densities and synapses formed by multisynaptic boutons (MSBs). One-way ANOVA indicated significant group effects (perforated: $F(3,26)=4.34$, $p=0.013$; MSB: $F(3,26)=3.88$, $p=0.020$). The post-hoc tests showed that there were significantly more perforated synapses ($p=0.0024$) and MSBs ($p=0.014$) in the moderately impaired CS group compared to NoCS. The severely impaired CS group was not significantly different from the impairment matched NoCS group (perforated: $p=0.34$; MSB: $p=0.16$) nor from Moderate CS on either measure.

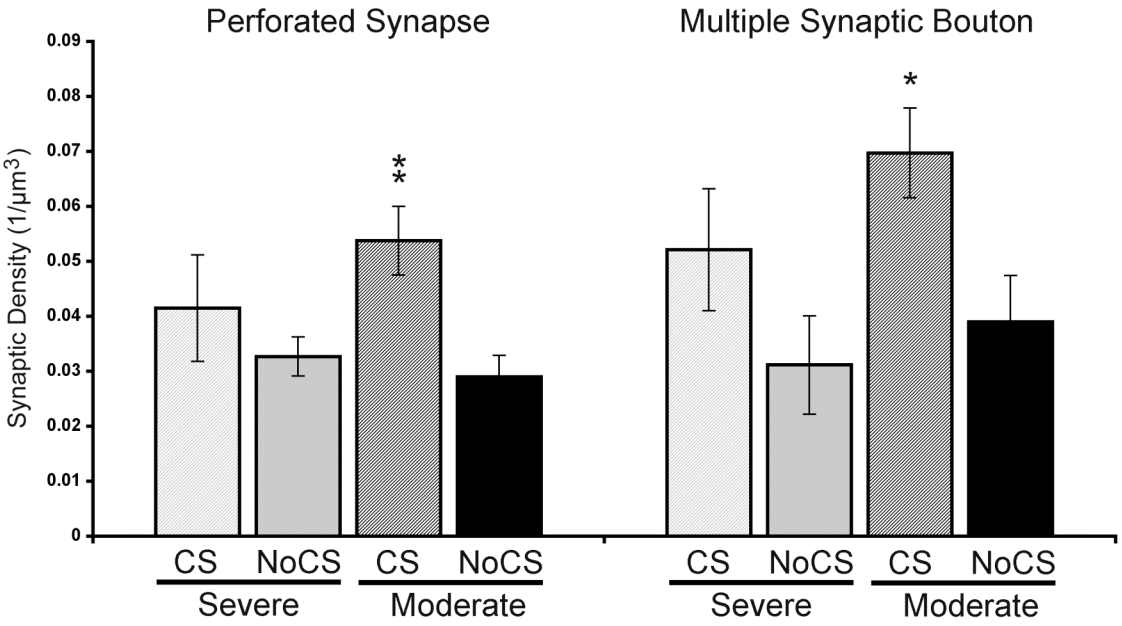

Figure 4.5 Cortical stimulation enhances the density of MSB and perforated synapses.

There were significant group effects of the density of both synapses with perforated post-synaptic densities and synapses formed by MSBs (p's<0.05). Post-hoc analysis showed that there were significantly more perforated synapses and MSBs in the moderately impaired CS group compared to NoCS. Data are means ±S.E.M. *p<0.05, **p<0.01.

4.4.3 Synaptic density is positively correlated with functional outcome.

Figure 4.6 illustrates that synaptic density was significantly correlated with reaching performance (% successful retrievals) in the post-CS training sessions (average of 2 days, $p = 0.005$, $r=0.50$). Final reaching performance was also significantly correlated with the density of perforated synapses ($p < 0.001$; $r = 0.61$) and MSBs ($p = 0.012$, $r = 0.45$) .

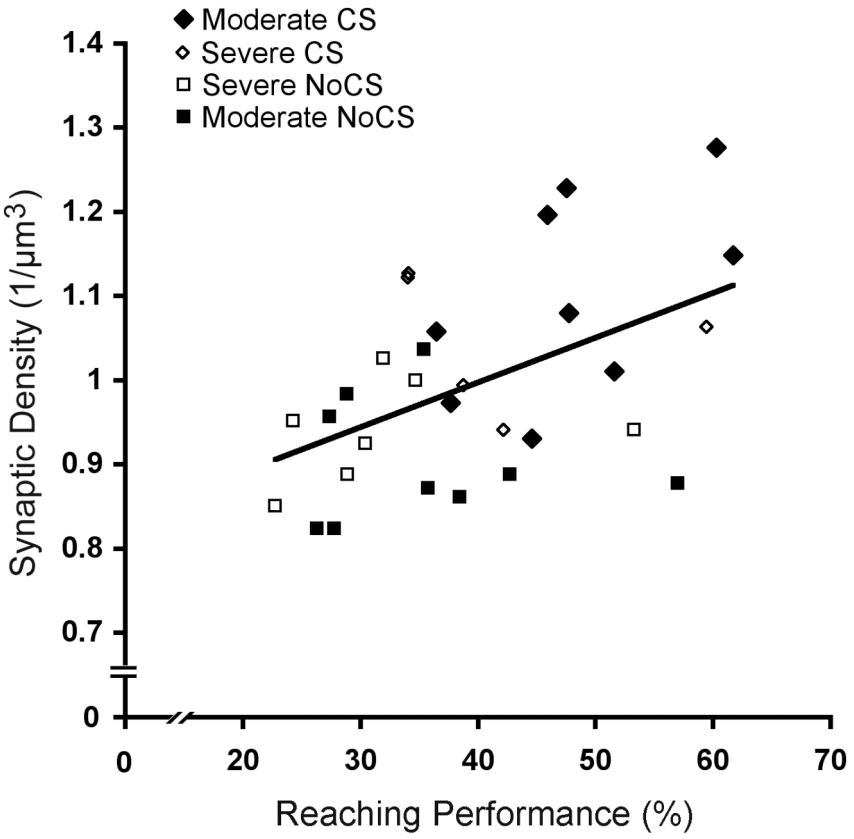

Figure 4.6 Synaptic density is correlated with reaching success on the single pellet reaching task.

Axodendritic synaptic density was positively correlated with reaching performance (% successful retrievals) in the last training session. ($p <0.01$, $r=0.50$)

4.5 DISCUSSION

Motor cortical stimulation (CS) during rehabilitative training improved reaching success in the single pellet retrieval task and was linked with an increased density of layer V synapses in the stimulated motor cortex in animals with both moderate and severe impairments. In moderately impaired animals, CS also significantly increased the density of synapses formed by multisynaptic boutons (MSBs) and synapses with perforated post-synaptic densities. The overall layer V synaptic density, as well as perforated and MSB synaptic densities, were positively correlated with functional outcome as measured by the final levels of reaching success.

Animals that received CS during motor training had a significantly greater density of synapses in layer V of peri-infarct motor cortex compared to animals receiving training alone, and the enhanced synaptogenesis was correlated with functional recovery as assessed by successful pellet retrieval. As discussed in Chapter 2, motor cortical plasticity plays an important role in the acquisition of a skilled reaching task in intact animals (Monfils *et al.*, 2005; Adkins *et al.*, 2006a). Neuronal structural plasticity in the motor cortex opposite the trained limb has been found following skilled reach training (Greenough *et al.*, 1985; Withers and Greenough, 1989; Kleim *et al.*, 2004). It is likely that synaptic plasticity in the perilesion cortex is also an important mediator of recovery from brain injury (Nudo, 2003; Kolb, 2003). As found in Chapters 2 and 3, rehabilitative skilled reaching with the impaired forelimb enhanced functional recovery and the synaptic structural plasticity of the perilesion cortex, and the enhanced functional recovery was blocked by local protein synthesis inhibition in the perilesion cortex.

Together, these findings suggest that plasticity in the stimulated region of cortex is likely to be an important contributor to the recovery of motor skill.

Previously, CS combined with post-lesion rehabilitative training was found to increase peri-infarct dendritic density (Adkins-Muir and Jones, 2003) and to induce functional alterations in the motor cortex, as revealed by expanded forelimb motor maps (Kleim *et al.*, 2003b; Plautz *et al.*, 2003) and neural potentiation, revealed as an increase in the late-component of motor evoked potentials (Teskey *et al.*, 2003) compared to unstimulated rehabilitation-only controls. The present finding of CS induced increases in synaptic density provides direct support that CS influences synaptic connectivity in the motor cortex and is consistent with the general hypothesis that positive modulation of synaptic plasticity mediates the behavioral improvements resulting from CS. Significant increases in the density of presumably more efficacious synapse subtypes also suggests that, following damage to the motor cortex, CS induces functional and structural alterations that may be related to enhanced synaptic efficacy within the remaining motor cortex (Teskey *et al.*, 2003; Monfils *et al.*, 2005). This synaptic plasticity may mediate the reinstatement of the neural integrity and reorganization of circuitry that underlies the functional improvements. Of course, this should not be taken to indicate that the motor cortex is the *only* region mediating functional recovery. Further research is needed to understand how this motor cortical plasticity may be coordinated with changes in connected cortical and subcortical regions.

The present findings support that CS can be used to promote functionally beneficial synaptic plasticity after brain damage. It was suggested by the present findings that the combination of task practice and cortical stimulation may aid in inducing greater

structural and functional plasticity within adjacent cortical and, possibly, corticospinal (see Brown *et al.*, 2003) pathways that lead to greater motor recovery following stroke. A better understanding of the specific neural mechanisms mediating these functional improvements may help to greatly improve motor "re-learning" after brain damage.

Chapter 5: General discussion

5.1 SUMMARY

The cerebral cortex is capable of changing its functional organization in response to experience, and this phenomenon is called cortical plasticity. Significant progress has been made in the past two decades to elucidate the factors that drive cortical plasticity in normal and injured brains (see review: Nudo, 2006 a,b). Skill acquisition (Greenough *et al.*, 1985; Kleim *et al.*, 2002; 2004), focal cortical injury (Hsu and Jone, 2005; 2006), electrical cortical stimulation (Adkins-Muir and Jones, 2003; see also Chapter 4), and exogenous neuromodulating drugs (Adkins and Jones, 2005; Gilmour *et al.*, 2005) are examples of factors that drive structural and functional alterations in the cerebral cortex, although the underlying mechanisms of these alterations are still poorly understood. Stroke is now the leading cause of long-term disability in industrialized countries. Exploration of the brain mechanisms involved during recovery from stroke is likely to yield information that can be used to promote better functional outcome. After focal motor cortical infarcts, reorganization of movement representations in the remaining motor cortex has been linked to both spontaneous recovery and recovery induced by rehabilitative training. In the first study of my disseration, it was found that motor rehabilitative training using the impaired forelimb on a skilled reaching task after a unilateral ischemic lesion improved forelimb functional outcome and facilitated synaptogenesis in the perilesion cortex. In the second study, it was found that the improved functional recovery seen following skilled rehabilitative training was disrupted

by focal transient protein synthesis inhibition in the perilesion cortex, consistent with the possibility that the structural plasticity in this area plays an important role in regaining function. In the last study, it was found that a therapy, cortical electrical stimulation, that enhances the efficacy of motor rehabilitation also enhances synaptic structural plasticity in the perilesion cortex, and the synaptic density in the perilesion cortex was positively correlated with post-rehabilitation reaching performance. This finding suggests that CS-induced functional improvements may be mediated by synaptic structural plasticity in the stimulated cortex.

Together, these dissertation studies indicate that, after a cortical lesion in rats, the enhancement of functional outcome by motor rehabilitation alone or in conjunction with other scientifically proven efficacious therapies can greatly facilitate synaptic structural plasticity in the perilesion cortex. Furthermore, these studies suggest that rehabilitation induced improvements in functional outcome are dependent upon the structural and functional integrity of the reorganized perilesion cortex.

5.2 POTENTIAL NEURAL SUBSTRATE OF THE BENEFICIAL EFFECTS OF REHABILITATION

Limited spontaneous motor recovery can occur after motor cortex injury, especially in regaining function in intensive task-specific skilled reaching. Explorations of the effects of therapeutic interventions on functional and structural plasticity in injured brains would provide mechanistic rationales for such interventions and help to develop more effective rehabilitation therapies. Following rehabilitative training in skilled reaching, the reorganization of motor representations in the perilesion cortex is induced and is thought

to contribute to relevant functional recovery (Castro-Alamancos and Borrell, 1995; Conner *et al.*, 2005). In humans, it was found that inactivation of the peri-lesion cortex by disruptive transcranial magnetic stimulation (TMS) reinstates functional deficits (Fridman *et al.*, 2004). Ablation of the reorganized cortex in rats (Castro-Alamancos and Borrel, 1995; Conner *et al.*, 2005) also disrupts regained function. These findings suggest that functional reorganization in the perilesion cortex after rehabilitative training plays an important role in the functional recovery of the impaired limb. The current findings (Chapter 2) indicate that skilled rehabilitative training using the impaired forelimb after a unilateral ischemic lesion improves behavioral outcome as measured by the percentage of successful retrievals in a skilled reaching task and facilitates greater density of total synapses along with efficacious synapse subtypes in layer V of the perilesion cortex compared to no rehabilitation control animals. Perforated synaptic density in layer V was significantly correlated with reaching success, while the MSB density in layer V was also found to be increased in animals with less abnormal grasping movements after rehabilitation. Carmichael (2006) indicates that cortical ischemic injury results in cascades of cellular and molecular changes in the remaining cortex, in which growth inhibition is suppressed for approximately one month after the lesion, and these changes are likely to be sensitive to and modulated by the effects of behavioral experience, such as rehabilitation. Sanes and Donoghue (2000) suggested that the intrinsic horizontal neuronal connections in primary motor cortex (M1), which interconnect large regions of MI and show activity-dependent plasticity, are a strong candidate substrate for map reorganization. The two studies above suggest that axonal sprouting in the perilesion cortex might arise from neurons in layer V of the remaining

motor cortex. Futhermore, focal protein synthesis inhibition in the perilesion cortex disrupts the functional recovery induced by rehabilitative training (Chapter 3). Together, these findings suggest that the functional recovery induced by motor rehabilitative training depends on the structural and functional integrity of the perilesion cortex.

In rodents, cellular and structural changes occur in the motor cortex contralateral and homotopic to a focal cortical lesion, including dendritic growth, synapse addition, and the up-regulation of NMDA receptor 1 (*e.g.* Jones, 1999; Jones *et al.*, 2003; Adkins *et al.*, 2004; Luke *et al.*, 2004; Hsu and Jones, 2005; 2006). An increase in neuronal growth and synaptogenesis-associated proteins has also been found in the contralesional cortex following focal cortical lesions (Stroemer *et al.*, 1995; McNeill *et al.*, 1999). It has been found in non-human primates that a focal ischemic lesion in the primary motor cortex hand area results in extensive ipsilateral axonal sprouting from the ventral premotor cortex to the somatosensory hand area (Dancause *et al.*, 2005). These findings indicate that neuroanatomical changes occur not only within the perilesion cortex after stroke, but also in more remote areas such as the intact ipsilesional adjacent cortex and the contralesional homotopic cortex. Plasticity in different remote areas might play a different role in functional recovery after rehabilitation; for example, the plasticity seen in the contralesional hemisphere might have a disruptive influence on the recovery of function (Murase *et al.*, 2004; Fregni *et al.*, 2005; Cramer and Crafton, 2005). In addition to intracortical and transcallosal connections, Conner *et al.* (2005) indicated that the basal forebrain cholinergic system is essential for functional recovery and cortical plasticity following rehabilitation in cortically injured rats. Further research is needed

to understand how this motor cortical plasticity may be coordinated with changes in connected cortical and subcortical regions.

As mentioned above, the lesion-induced plasticity in the contralesional, intact hemisphere might have a disruptive influence on functional recovery in the contralesional, impaired body side. Recent studies on interhemispheric interaction and the potentially disruptive influence of the contralesional hemisphere help to provide a more complete account of the behavioral and neuroanatomical responses to cortical stroke.

5.3 IMPLICATION OF CURRENT LESION MODEL FOR HUMAN STROKE

The most important findings in this dissertation are (1) that after a cortical lesion in rats, motor rehabilitation can greatly enhance synaptic structural plasticity in the perilesion cortex and facilitate improvements in functional outcome, and (2) that these improvements are dependent upon the structural and functional integrity of the reorganized perilesion cortex. In the animal model employed for this dissertation, endothelin-1 (ET-1), a potent and long-acting vasoconstrictor, was used to induce focal ischemic lesions within the sensorimotor cortex (SMC) of young adult rats that had received early skilled reach training. The surface application of ET-1 has been shown to reliably produce a local dose dependent ischemic lesion with minimal tissue edema in the SMC of rats (Adkins *et al.*, 2004; Hsu and Jones, 2005; 2006). Furthermore, after ET-1 application, the compromised tissue undergoes a period of reperfusion, a phenomenon which has been shown to exacerbate injury after a stroke. An important factor that must be considered with this particular type of lesion is the finding that ET-1 application also

induces astrocytic growth and appears to facilitate axonal sprouting after spinal cord injuries, which might interfere with the interpretation of neural repair experiments (Uesugi *et al.*, 1996). Nevertheless, the current findings of structural plasticity in the perilesion cortex following ET-1 induced ischemic lesions seem compatible with the functional reorganization found in the perilesion cortex following cauterization lesions (Nudo *et al*., 1996b) and electrolytic lesions (Conner *et al.*, 2005).

Some other factors might also limit the applications of the current findings for other animal models of brain injury and human stroke. For example, the model used for brain injury in this dissertaion is ischemic stroke, and the ways in which the damage caused by stroke evolve (*e.g.* the progression of stress responses and the death of ischemic cells following the initial ischemic insult) and the characteristics of reperfusion and reperfusion-induced further damage might be different from those seen after traumatic brain injury, which occurs when a sudden trauma causes brain damage. The location and size of the lesions in the current model are confined within the sensorimotor cortex. In other stroke models, such as middle cerebral artery occlusion (MCAO), damage is seen in other cortical areas and often in subcortical areas, and these differences in lesion site might influence perilesional structural plasticity differently. In human strokes, lesions are more variable, often including multiple lesion sites in cortical areas, subcortical areas, or in both. Also, human strokes can vary greatly in size with some, such as lacunar strokes, being relatively small while others are large infarcts inclusive of gray and white matter. Usually, but not always, larger cerebral lesions result in severe behavioral impairments and poorer functional reorganization in the remaining cerebrum (see review: Teasell *et al.*, 2006). In the present findings, though the lesions in the

experiments described in Chapter 4 were relatively larger than the ones in Chapter 2, it was still found that synaptic plasticity occured in the perilesion cortex, suggesting that structural plasticity does still occur in the perilesion site following relatively larger ischemic lesions.

The timing of rehabilitation is also an important factor that affects the improvement of functional recovery and structural plasticity in the perilesion cortex. The onset of rehabilitation for the experiments described in Chapters 2 and 4 was four days and approximately two weeks after surgery, respectively. This difference in the onset of rehabilitation might be part of the reason why moderately impaired rats which received rehabilitation only (Moderate NoCS group) in Chapter 4 showed more limited improvement of skilled reach training after rehabilitation. Biernaskie *et al.* (2004) also showed that rehabilitation initiated 5 days after focal ischemic brain injury resulted in better improvement in functional recovery compared to rehabilitation initated 30 days after brain injury in rats. These findings suggest that the brain displays an enhanced sensitivity to rehabilitative experience early after stroke and this sensitivity declines with time. A topic for future experiments is whether the functional improvements and synaptic plasticity in perilesion cortex found in the present studies can be induced by motor skill training that is initiated much later after the injury.

Finally, the subjects' ages also affect the outcome of functional recovery and structural plasticity in the perilesion cortex. In the clinical field, most patients that suffer from stroke are elders. It was found that neuroplastic responses are altered in the aged brain (Nieto-Sampedro and Niet-Diaz, 2005), and, while ischemic injury in young adult rats results in increases in neurogenesis, these increases are greatly reduced in aged

rats (Jin *et al.*, 2004). Although research on the effects of rehabilitation after brain damage in aged animals is scant, healthy aged animals have been shown to benefit from complex motor skills training (Churchill *et al.*, 2003) and exposure to complex social environments (Greenough *et al.*, 1986), suggesting that the beneficial effect of rehabilitation might still occur in aged animals with brain injury.

5.4 DISRUPTIVE INFLUENCES OF THE CONTRALESIONAL HEMISPHERE

The cortex contralateral and homotopic to unilateral cortical lesions in rats has been found to undergo neuronal structural plasticity as described in the previous section. In humans who have experienced a unilateral stroke, previous studies have found increased excitability (Leipert *et al.*, 2000) in the contralateral cortex. Some of these contralesional changes are associated with enhanced ipsilesional function. Several studies have found that small lesions of the sensorimotor cortex (SMC) enhance subsequent skill learning in the ipsilesional forelimb in rats (Allred and Jones, 2004; Bury and Jones, 2002; Luke *et al.*, 2004; Hsu and Jones, 2005; 2006). Though these contralesional effects may facilitate behavioral compensation with the ipsilesional body side, it might also encourage animals to neglect to use their impaired forelimbs. Furthermore, increased contralesional activity is associated with poorer functional recovery of the impaired body side (*e.g.*, Ward and Cohen, 2004). In humans, several studies have shown that unilateral infarcts create interhemispheric functional imbalances that worsen performance in the impaired limb (*e.g.*, Fregni *et al.*, 2005; Cramer and Crafton, 2005). For example, Murase *et al.* (2004) found that the contralesional cortex

has an abnormal inhibitory effect during the process of generating voluntary movements with the paretic hand.

In both healthy humans and stroke patients, the production of transient virtual motor cortical lesions using repetitive transcranial magnetic stimulation (rTMS) enhances motor performance in the ipsilesional hand (Kobayashi *et al.*, 2004; Takeuchi *et al.*, 2005). Moreover, in stroke patients, Floel *et al.* (2004) showed that ischemic nerve block of the less-affected hand improved performance on a finger tapping task with the paretic hand. The studies above suggest that a unilateral cerebral lesion can lead to an imbalance between the two hemispheres that favors the contralesional cortex, and that reducing activity in one hemisphere or one body side can improve function in the other.

5.5 THE ROLE OF ADJUVANT THERAPIES IN REHABILITATION

The mechanisms that underlie cortical electrical stimulation's beneficial effects on recovery of behavioral function are still not well known. Cortical stimulation in conjunction with motor rehabilitative training may cause a release from diaschisis or enhance neural transmission within affected motor systems. Recent animal studies have shown that rehabilitation in conjunction with electrical stimulation of the perilesion cortex enhance recovery in rats (Adkins-Muir and Jones, 2003; Kleim *et al.*, 2003b; Teskey *et al.*, 2003), monkeys (Plautz *et al.*, 2003), and humans (Brown *et al.*, 2006). In rats with focal cortical lesions, it has been found that electrical cortical stimulation during rehabilitation increases dendritic density in layer V of the cortex lateral to the lesions (Adkins-Muir and Jones, 2003). It has also been found that, in comparison to control animals that received rehabilitation only, cortical electrical stimulation during

rehabilitation enhances functional alterations in the remaining motor cortex, as revealed by expanded forelimb motor maps (Kleim *et al.*, 2003b; Plautz *et al.*, 2003), and enhances neural potentiation (Teskey *et al.*, 2003), which is measured as an increase in the late-component of motor evoked potentials in the perilesion cortex. In the present study (Chapter 4), it was found that cortical electrical stimulation enhanced synaptic structural plasticity in the perilesion cortex, and synaptic density in the perilesion cortex was positively correlated with post-rehabilitation reaching performance. These findings suggest that plasticity in the stimulated region of cortex is likely to be an important contributor to the recovery of motor skill.

D-amphetamine (d-AMPH) coupled with rehabilitation has been shown to enhance the recovery of behavioral functions in rats (Feeney *et al.*, 1982; Adkins and Jones, 2005; Gilmour *et al.*, 2005) and non-human primates (Barbay *et al.*, 2006). However, in recent clinical trials not all of the participants experienced beneficial effects from this adjuvant therapy (Gladstone *et al.*, 2006; Platz *et al.*, 2005). Further investigations are necessary in order to elucidate how d-AMPH alters functional and structural components in the central nervous system after stroke.

The disruptive influences of the intact contralesional hemisphere on the lesion hemisphere and impaired body side have been discussed in the previous section. Some studies have indicated that reducing activity in one hemisphere or one body side can improve function in the other (Floel *et al.*, 2004; Takeuchi *et al.*, 2005). In rodent models, constraint of the intact forelimb coupled with rehabilitation exercises improved motor function and decreased the volume of brain injury after striatal hemorrhagic stroke (DeBow *et al.*, 2003). The Extremity Constraint Induced Therapy Evaluation

(EXCITE) study in humans suggests that, in moderately impaired patients who maintain some hand and wrist movement at one year after a stroke episode, treatment with constraint-induced movement therapy (CIMT) for two weeks can improve upper extremity function, and these improvements persist for at least 1 year. This therapeutic intervention was developed based on the theory of learned nonuse of the paretic arm in the deafferented monkey model (Taub and Uswatt, 2006), though the mechanisms that underlie this intervention are still not well known.

5.6 CONCLUSION

Rehabilitative training can greatly enhance functional outcome and synaptic structural plasticity in the perilesion cortex, and the improved functional outcome is dependent upon the structural and functional integrity of the reorganized perilesion cortex. To my own knowledge, this set of studies is the first evidence showing an increase in synaptic density and efficacious synaptic subtypes, as measured by quantitative electromicroscopy, in the perilesion cortex of rodents that have undergone skilled rehabilitative training. These studies further support that synaptic plasticity in the perilesion cortex might be the underlying mechanism for the improvement in functional outcome that follows rehabilitation with adjuvant therapy. Finally, the finding that the improved function that follows rehabilitation can be disrupted by an acute alteration in the structural and functional integrity of the perilesion cortex is the first demonstration that synaptic plasticity in the perilesion cortex plays an important role in recovered function. Further investigation is necessary in order to determine what mechanisms, such as which proteins and what changes in the expression of specific receptors or trophic

factors, are responsible for the structural plasticity in the reorganized motor cortex that drives the behavioral improvements that follow rehabilitation.

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